# KAPA LTP Library Preparation Kit Illumina® platforms

KR0453 – v3.13

This Technical Data Sheet provides product information and a detailed protocol for the KAPA LTP Library Preparation Kit (Illumina® platforms), product codes KK8230, KK8231, KK8232 and KK8233.

## Contents



## Kit Codes and Components

| | | |
|---|---|---|
| **KK8230**<br>**KK8231**<br>10 libraries | KAPA End Repair Buffer (10X) | 100 µl |
| | KAPA End Repair Enzyme Mix | 50 µl |
| | KAPA A-Tailing Buffer (10X) | 50 µl |
| | KAPA A-Tailing Enzyme | 30 µl |
| | KAPA Ligation Buffer (5X) | 100 µl |
| | KAPA DNA Ligase | 50 µl |
| | KAPA PEG/NaCl SPRI® Solution | 5 ml |
| | KAPA HiFi HotStart ReadyMix (2X)* | 250 µl |
| **KK8232**<br>**KK8233**<br>50 libraries | KAPA End Repair Buffer (10X) | 500 µl |
| | KAPA End Repair Enzyme Mix | 250 µl |
| | KAPA A-Tailing Buffer (10X) | 250 µl |
| | KAPA A-Tailing Enzyme | 150 µl |
| | KAPA Ligation Buffer (5X) | 500 µl |
| | KAPA DNA Ligase | 250 µl |
| | KAPA PEG/NaCl SPRI® Solution | 20 ml |
| | KAPA HiFi HotStart ReadyMix (2X)* | 1.25 ml |

*KK8231 and KK8233 are available for PCR-free workflows and do not contain KAPA HiFi HotStart ReadyMix for library amplification.

## Quick Notes

- The protocol provided in this document is a generic prototype, and may require additional tailoring and optimization.
- The process workflow (p. 8) provides an overview of the library construction process and options for size selection.
- The **KAPA NGS Library Preparation Technical Guide** contains more detailed information about library construction parameters, and may facilitate protocol development and optimization.
- Separate, concentrated enzyme formulations and reaction buffers for end repair, A-tailing, and ligation provide the best combination of product stability, convenience, and efficiency.
- Adapters and PCR primers are not supplied with this kit, and can be obtained from any reputable oligonucleotide vendor.
- SPRI® beads are not included in the kit, but the PEG/NaCl SPRI® Solution required for "with-bead" reaction cleanups is provided.

## Product Description

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

1. **End repair,** which produces blunt-ended, 5'-phosphorylated 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.
4. **Library amplification (optional),** which employs PCR to amplify library fragments carrying appropriate adapter sequences on both ends.

The kit has been validated for library construction from 100 ng – 5 µg of human genomic DNA for whole-genome shotgun sequencing or targeted sequencing by solution hybrid selection (capture). For smaller genomes, or lower complexity samples, such as ChIP DNA, amplicons, or cDNA (for RNA-seq), successful library construction has been achieved from low nanogram to picogram quantities (≥100 pg) of input DNA.

The kit provides all of the enzymes and buffers required for library construction and amplification, but does not include adapters, PCR primers, or SPRI® beads. Enzyme formulations and reaction buffers for end repair, A-tailing, and ligation are supplied in convenient, concentrated formats.

Efficient, cost-effective reaction cleanups and higher recovery of input DNA are achieved through implementation of the "with-bead" strategy developed at The Broad Institute of MIT & Harvard and Foundation Medicine[1]. The kit includes PEG/NaCl SPRI® (Solid Phase Reversible Immobilization) Solution for this purpose.

In order to maximize sequence coverage uniformity, it is critical to minimize library amplification bias. KAPA HiFi DNA Polymerase has been designed for low-bias, high-fidelity PCR, and is the reagent of choice for NGS library amplification[2, 3, 4]. KAPA LTP Library Preparation Kits (KK8230 and KK8232) include KAPA HiFi HotStart ReadyMix (2X), a ready-to-use PCR mix comprising all the components for library amplification, except primers and template. Kits without an amplification module (KK8231 and KK8233) are available for PCR-free workflows. These kits can 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 libraries that have undergone bisulfite-treatment.

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 – 11 (2012).
4. Quail M.A. *et al. BMC Genomics* **13**: 341 (2012).

## Product Applications

The KAPA LTP Library Preparation Kit is ideally suited for low-throughput, manual NGS library construction workflows that involve end repair, A-tailing, adapter ligation, and library amplification (optional). The protocol may be adapted for incorporation into workflows for a wide range of NGS applications, including:

- Whole-genome shotgun sequencing
- Targeted sequencing by solution hybrid selection (i.e. exome or custom capture using the Roche Nimblegen™, Agilent SureSelect, Illumina® TruSeq™, or IDT xGen™ Lockdown™ Probes systems)
- ChIP-seq
- RNA-seq
- Methyl-seq (in combination with the KAPA HiFi HotStart Uracil+ ReadyMix)

Specific guidelines for the construction of libraries for target enrichment using the Roche Nimblegen™ SeqCap EZ system may be found in Appendix 1.

## 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 Library Preparation Kits are shipped on dry ice or ice packs, depending on the country of destination. Upon receipt, immediately store enzymes and reaction buffers at -20 °C in a constant-temperature freezer. The PEG/NaCl SPRI® Solution should be protected from light, and stored at -20 °C. For short-term use, the PEG/NaCl SPRI® Solution may be stored at 4 °C for up to 2 months. 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 components have been fully thawed and thoroughly mixed before use. Keep all enzyme components and master mixes on ice as far as possible during handling and preparation. KAPA HiFi HotStart ReadyMix (2X) contains isostabilizers and may not freeze completely, even when stored at -20 °C. Nevertheless, always ensure that the KAPA HiFi HotStart ReadyMix is fully thawed and thoroughly mixed before use. PEG/NaCl SPRI® Solution does not freeze at -20 °C, but should be equilibrated to room temperature and thoroughly mixed before use.

### 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 **support@kapabiosystems.com** 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 a generic prototype, and there are many parameters which may be adjusted 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 **support@kapabiosystems.com** for further guidelines when designing or optimizing your library construction workflow.

### Reaction setup

While this kit is intended for low-throughput, manual library construction, the protocol is designed to be automation-friendly in order to facilitate the transition to automation should throughput requirements grow over time. For this reason, and to enable a streamlined "with-bead" strategy, reaction components are combined into master mixes, rather than dispensed separately into individual reactions. When processing multiple samples, prepare 5 – 10% excess of each master mix, to allow for small inaccuracies during dispensing.

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 free of DNAses, RNAses, and nucleases. Low DNA-binding plastics are recommended. When selecting the most appropriate plasticware for your workflow, consider compatibility with:

- the magnet used during SPRI® bead manipulations.
- vortex mixers and centrifuges, where appropriate.
- 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 hours, depending on the specific workflow, number of samples being processed, and experience. If necessary, the protocol may be paused safely after any of the bead cleanup steps, as described below:

- After the end repair cleanup (Steps 3.1 – 3.13), resuspend the washed beads in 20 µl of 1X A-Tailing Buffer (without enzyme; Step 4.1), and store the reactions at 4 °C for up to 24 hours.
- After the A-tailing cleanup (Steps 5.1 – 5.13), resuspend the washed beads in 20 µl of 1X Ligation Buffer (without enzyme or adapter; Step 6.1), and store the reactions at 4 °C for up to 24 hours.
- After the first post-ligation cleanup (Steps 7.1 – 7.13), resuspend the washed beads in the appropriate volume of 10 mM Tris-HCl (pH 8.0) as outlined in Step 7.14, and store the reactions at 4 °C for up to 24 hours.

DNA solutions containing 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 4B and 5B on p. 10). If the protocol was paused after the first post-ligation cleanup, continue directly with the second post-ligation cleanup (Step 7.16), dual-SPRI® size selection (Step 8.1), or size selection using an alternative method.

Adapter-ligated DNA that has been completely cleaned up or size-selected can be stored at 4 °C for one week, or at -20 °C for at least one month before amplification, target enrichment, and/or sequencing. To avoid degradation, always store DNA in a buffered solution (10 mM Tris-HCl, pH 8.0) and minimize the number of freeze-thaw cycles.

### Paramagnetic SPRI® beads and reaction cleanups

- Cleanups should be performed in a timely manner to ensure that enzyme reactions do not proceed beyond optimal incubation times.
- This protocol has been validated using Agencourt® AMPure® XP reagent (Beckman Coulter, part number A63880, A63881, or A63882). Solutions and conditions for DNA binding and size selection may differ if other beads are used.
- Observe all the manufacturer's storage and handling recommendations for AMPure® XP reagent.
- Beads will settle gradually; always ensure that they are fully resuspended before aspirating AMPure® XP reagent.
- The incubation times provided for reaction cleanups and size selection are guidelines only, and should be modified/optimized according to your current protocols, previous experience, and specific equipment and samples in order to maximize library construction efficiency and throughput.
- The time required to completely capture magnetic 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. Where possible, use a wash volume that is equal to the volume of sample plus AMPure® XP reagent or PEG/NaCl SPRI® Solution.

### Paramagnetic SPRI® beads and reaction cleanups (continued)

- It is important to remove all ethanol before proceeding with subsequent reactions. However, over-drying of beads may make them difficult to resuspend, and may result in a dramatic loss of DNA. With optimized pipetting, 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). Elution of DNA in PCR-grade water is not recommended, as DNA is unstable in unbuffered solutions.

### Input DNA and fragmentation

- This protocol has been validated for library construction from 100 ng – 5 µg of appropriately fragmented, double-stranded DNA. However, libraries can be prepared from lower input amounts if the sample represents sufficient copies to ensure the requisite coverage and complexity in the final library. Successful library construction has been achieved from <100 pg of ChIP DNA, low nanogram quantities of cDNA or microbial DNA, and 1 – 10 ng of high-quality human or mouse genomic DNA.
- The above typically refers to the input into the end repair reaction. If input DNA is quantified before fragmentation, and/or fragmented DNA is 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.
- The proportion of fragmented 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 recovered as adapter-ligated molecules, whereas the recovery typically ranges from 0.5 to 15% for libraries constructed from 100 pg – 100 ng DNA. These figures apply to high quality DNA and can be significantly lower for DNA of lower quality, e.g. FFPE samples. Workflows that contain additional SPRI® cleanups or size selection prior to library amplification are likely to result in a lower yield of adapter-ligated molecules.
- Solutions containing high concentrations of EDTA and strong buffers may negatively affect the end repair reaction, and should be avoided. If fragmented DNA will not be processed (i.e. subjected to cleanup or size selection) prior to end repair, DNA should be fragmented in 10 mM Tris-HCl (pH 8.0 or 8.5) with 0.1 mM EDTA. Fragmentation in water is not recommended.
- In some circumstances it may be convenient to fragment input DNA in 1X KAPA End Repair Buffer, in which case the end repair reaction setup should be adjusted accordingly. Please contact **support@kapabiosystems.com** for more information.

### Cleanups after end repair and A-tailing

- This protocol provides for 1.7X – 1.8X cleanups after end repair and A-tailing. This ratio of PEG/NaCl SPRI® Solution to sample volume will retain most DNA fragments larger than ~75 bp. If you wish to retain very small DNA fragments, the PEG/NaCl SPRI® 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, 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 should be prepared in 500 µl PCR tubes or 1.5 ml microtubes, or the end repair reaction should be scaled down. Please contact **support@kapabiosystems.com** for more information.

### Adapter design and concentration

- This protocol has been validated using standard, indexed Illumina® TruSeq™ "forked" adapters, but the kit is compatible with other adapters of similar design.
- Adapter concentration affects ligation efficiency, as well as adapter and adapter-dimer carry-over in post-ligation cleanups. The optimal adapter concentration for your workflow represents a compromise between cost and the above factors. Your choice of post-ligation cleanup and size-selection options should be informed by your choice of adapter concentration. Please refer to the next subsection for more details.
- Ligation efficiency is robust for adapter:insert molar ratios ranging from 10:1 to 50:1. As a general guideline, we recommend an adapter:insert molar ratio of ~10:1, for libraries constructed from ≥100 ng fragmented DNA. This translates to different final adapter concentrations for libraries with different size distributions (see Table 1 on the next page). An adapter:insert molar ratio >10:1 **may** be beneficial for libraries constructed from lower amounts of input DNA.
- While it is not necessary to adjust adapter concentrations to accommodate moderate sample-to-sample variations, we recommend using an adapter concentration that is appropriate for the range of input DNA concentrations.
- The best way to accommodate different adapter concentrations within a batch of samples processed together, is to vary the concentration 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 less compatible with higher throughput or automated workflows.

Table 1. Recommended adapter concentrations.

| Insert DNA per 50 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 | 0.7 µM |
| 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 |

## Post-ligation cleanup

- It is important to remove excess unligated adapter and adapter-dimer molecules from the library prior to library amplification or cluster generation.
- While a single SPRI® bead cleanup removes most unligated adapter and adapter-dimer, a second SPRI® bead cleanup is recommended to eliminate any remaining 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 size selection is carried out between adapter ligation and library amplification (or clustering), a single post-ligation cleanup with SPRI® beads (1X) is usually sufficient prior to size selection. If no post-ligation size selection is carried out, two consecutive 1X SPRI® bead cleanups are recommended.
- 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 an optimized 50 µl library amplification reaction should yield ~1 µg of DNA, and can accommodate a maximum of 20 µl template DNA, an elution volume of 22 – 32 µl is recommended.
  - If proceeding with size selection, elute the library DNA in an appropriate volume according to the size selection method of choice. For the dual-SPRI® size selection procedure described here, beads have to be resuspended in a final elution volume of 100 µl.

## Size selection

- Size selection requirements vary widely according to specific applications. Depending on preference, the dual-SPRI® size selection procedures presented in this protocol may be omitted entirely, modified, or replaced with alternative size selection procedures. Size selection may be carried out at alternative points in the overall workflow, for example:
  - prior to end repair of fragmented DNA.
  - immediately before library amplification (as outlined in the protocol that follows).
  - after library amplification.
- Size selection inevitably leads to a loss of sample material. Depending on the details, these losses can be dramatic (>80%), and may significantly increase the number of amplification cycles needed 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 carefully 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.
- 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 artefacts, and typically contain authentic library molecules of the appropriate length. To eliminate these artefacts, optimization of library amplification reaction parameters (cycle number and/or primer concentration), rather than post-amplification size selection, is recommended. See Library amplification on pp. 6 – 7 for more information.

### Size selection (continued)

- KAPA Ligation Buffer contains high concentrations of PEG 6000, which will interfere with efficient dual-SPRI® size selection and can affect the efficiency of other size selection techniques if not removed. It is therefore important to perform at least one post-ligation SPRI® bead cleanup (1X) prior to dual-SPRI® or any other size selection method.
- The dual-SPRI® size selection procedure described in Section 8 of the protocol is designed for selection of adapter-ligated fragments approximately 250 – 450 bp in size. Consult the **KAPA NGS Library Preparation Technical Guide** if you wish to select a different range of fragment sizes.
- "Forked" adapters with long single-stranded arms (such as the TruSeq™ design) noticeably affect the size-dependent binding of DNA to SPRI® beads, as well as the apparent size of fragments determined by some electrophoresis instruments (e.g. those employing microfluidic chips and tapes). Size selection parameters will therefore require optimization depending on a number of factors, including:
  - The design of the adapters used.
  - The point at which size selection is applied in the protocol; size selection of dsDNA fragments prior to library construction or after amplification may require different parameters than those used for post-ligation size selection of fragments carrying forked adapter ends.
- Dual-SPRI® size selection is sensitive to multiple factors that are beyond the scope of this document. The **KAPA NGS Library Preparation Technical Guide** contains additional guidelines for the optimization of dual-SPRI® size selection parameters. Any dual-SPRI® size selection protocol should be carefully optimized and validated before it is used for precious samples.

### 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 DNA Polymerase has 5'→3' polymerase and 3'→5' exonuclease (proofreading) activities, 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 DNA Polymerase is $2.8 \times 10^{-7}$ errors/base, equivalent to 1 error in $3.5 \times 10^{6}$ nucleotides incorporated.
- 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 are typically comprised of library molecules of the desired length, which are individualized during denaturation prior to cluster amplification or probe hybridization. Since these heteroduplexes contain significant portions of single-stranded DNA, over-amplification leads to the under-quantification of library molecules with assays employing dsDNA-binding dyes. qPCR-based library quantifications methods, such as the KAPA Library Quantification assay, quantify DNA by denaturation and amplification, thereby providing an accurate measure of the amount of adapter-ligated molecules in a library, even if the library was over-amplified.

  Please refer to the **KAPA NGS Library Preparation Technical Guide** 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 artefacts 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 enrichment or sequencing).
- If cycled to completion (**not recommended**) a single 50 µl library amplification PCR, performed as described in Section 9, can produce 8 – 10 µg (160 – 200 ng/µl) of amplified library. To minimize over-amplification and associated undesired artefacts, the number of amplification cycles should be optimized to produce an amplified library with a concentration in the range of 10 – 30 ng/µl (0.5 – 1.5 µg of PCR product per 50 µl reaction).
- 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. Using a simple calculation (for exponential amplification), the number of amplification cycles needed to achieve a specific yield of amplified library may be predicted theoretically (see Table 2 on the next page).

## Library amplification (continued)

- The **actual** optimal number of amplification cycles may be 1 - 3 cycles higher, particularly for libraries constructed from FFPE DNA or other challenging samples, or libraries with a broad fragment size distribution.

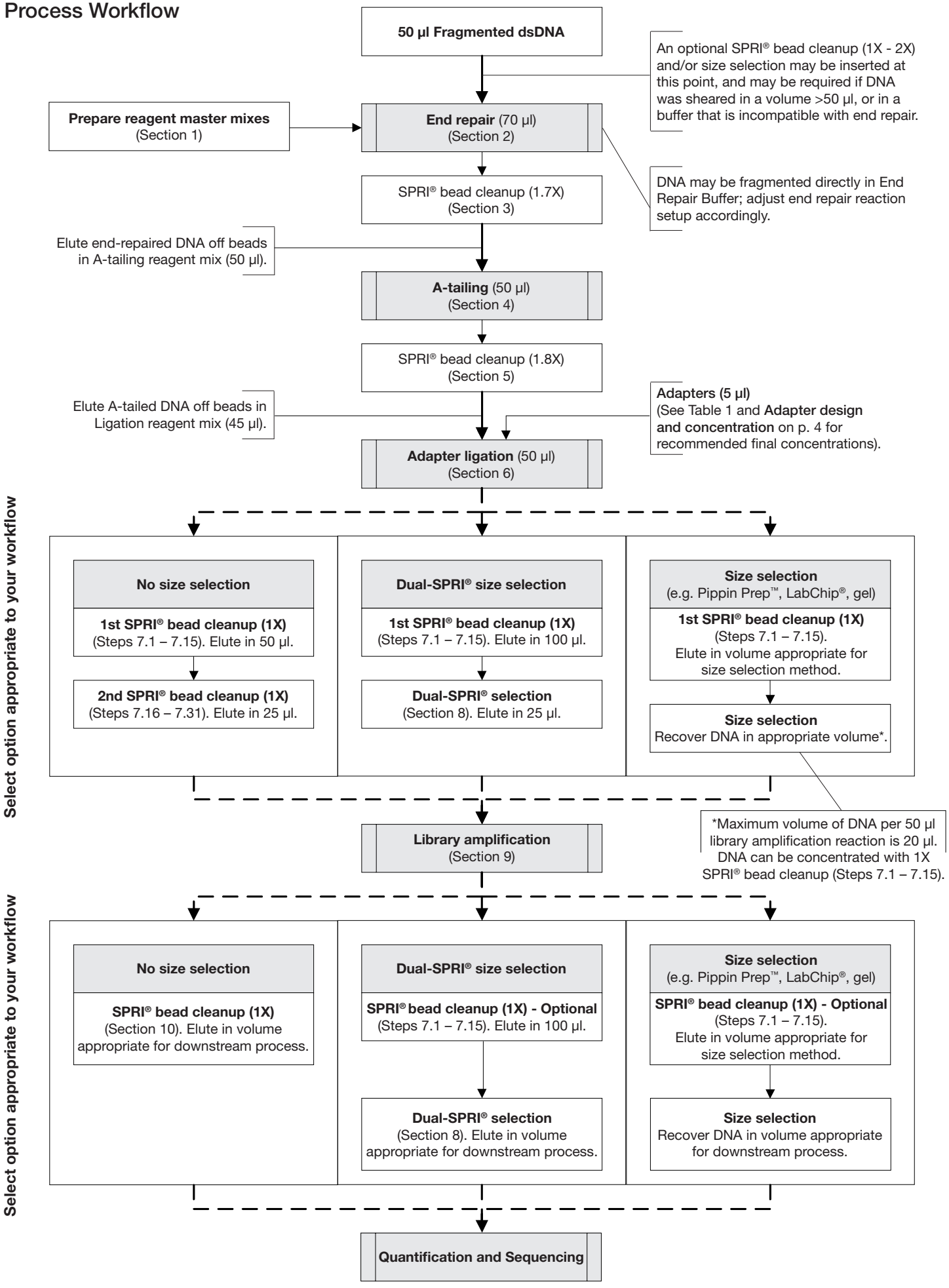
**Table 2. Theoretical number of cycles required to achieve 0.5 – 1.5 µg (10 – 30 ng/µl) library in a standard 50 µl KAPA HiFi HotStart ReadyMix library amplification reaction, starting from different amounts of template DNA.**

| Template DNA | Number of cycles |
|---|---|
| 1 ng | 10 – 11 |
| 5 ng | 7 – 8 |
| 10 ng | 6 – 7 |
| 25 ng | 5 – 6 |
| 50 ng | 4 – 5 |
| 100 ng | 3 – 4 |
| 250 ng | 2 – 3 |
| 500 ng | 0 – 2 |

## 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 the final or pre-capture library should be confirmed with an electrophoretic method, whereas 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.
  - that were constructed with full-length adapters after ligation, size selection, pre-capture amplification.
- Once a library construction workflow has been optimized, and consistently yields the desired amount of amplified library, it is typically not necessary to perform in-process quality control. However, qPCR-based quantification of libraries after adapter ligation or prior to library amplification can provide useful data for optimization or troubleshooting.
- Accurate quantification of DNA at this stage will allow you to evaluate the efficiency of:
  - the process from end repair to 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 of the process, qPCR quantification before and after size selection may also be helpful to define the relative benefit of size selection, and the loss of material associated with the process.
- Electrophoretic evaluation of libraries after adapter ligation or before library amplification may be informative, but remember that unamplified libraries prepared with "forked" adapters will appear to have a larger, wider or bimodal fragment size distribution than the corresponding amplified libraries (refer to Size selection on pp. 5 – 6). The difference in overall appearance and fragment size distribution of an unamplified *vs* corresponding amplified library varies depending on the electrophoretic system used. To accurately determine the size distribution of an unamplified library, an aliquot of the library may be subjected to one or two cycles of amplification (to ensure that adapters are fully double-stranded) prior to electrophoretic analysis. Alternatively, useful information may also be obtained by electrophoretic analysis of library quantification products generated with the KAPA Library Quantification Kit.

## Process Workflow



## Library Construction Protocol

### 1. Reagent preparation

For maximum stability and shelf-life, the enzyme formulations 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 3 – 6.

Additional reagents required for the KAPA LTP library preparation protocol are listed in Table 7.

Master mixes may be constituted with varying proportions of the total final water requirement. In the examples given in the tables below, all the required water is included in each master mix, allowing the entire reaction mix to be added 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 can be added to the beads, for storage at 4 °C for up to 24 hours. To resume library construction, prepare the master mix with the remaining volume of water and reaction buffer, and the required volume of enzyme. Recommendations on how to formulate master mixes at safe stopping points are provided in Tables 4B and 5B.

Master mixes for end repair, A-tailing and ligation may be prepared immediately before use, or stored for up to 1 week at 4 °C or 2 weeks at -20 °C. Master mixes stored at -20 °C are stable through three freeze-thaw cycles.

Table 3. Composition of the end repair reaction

| Component | 1 Library | 8 Libraries (incl. 5% excess) | 48 Libraries (incl. 5% excess) |
|---|---|---|---|
| **End Repair Master Mix:** | | | |
| Water | 8 µl | 67 µl | 403 µl |
| 10X KAPA End Repair Buffer | 7 µl | 59 µl | 353 µl |
| KAPA End Repair Enzyme Mix | 5 µl | 42 µl | 252 µl |
| **Total master mix volume** | **20 µl** | **168 µl** | **1 008 µl** |
| **Other components of the final reaction:** | | | |
| Fragmented DNA | 50 µl | | |
| Balance of water required | 0 µl | | |
| **Total reaction volume** | **70 µl** | | |

Table 4A. Composition of the A-tailing reaction (standard, uninterrupted protocol)

| Component | 1 Library | 8 Libraries (incl. 5% excess) | 48 Libraries (incl. 5% excess) |
|---|---|---|---|
| **A-Tailing Master Mix:** | | | |
| Water | 42 µl | 353 µl | 2 117 µl |
| 10X KAPA A-Tailing Buffer | 5 µl | 42 µl | 252 µl |
| KAPA A-Tailing Enzyme | 3 µl | 25 µl | 151 µl |
| **Total master mix volume** | **50 µl** | **420 µl** | **2 520 µl** |
| **Other components of the final reaction:** | | | |
| End repaired DNA with beads | 0 µl | | |
| Balance of water required | 0 µl | | |
| **Total reaction volume** | **50 µl** | | |

Table 4B. Composition of the A-tailing reaction if process is stopped after end repair.

| Component | 1 Library | 8 Libraries (incl. 5% excess) | 48 Libraries (incl. 5% excess) |
|---|---|---|---|
| **Resuspend beads in mix of:** | | | |
| Water | 18 µl | 151 µl | 907 µl |
| 10X KAPA A-Tailing Buffer | 2 µl | 17 µl | 101 µl |
| KAPA A-Tailing Enzyme | 0 µl | 0 µl | 0 µl |
| **Total volume of mix** | **20 µl** | **168 µl** | **1 008 µl** |
| **To resume, add mix of:** | | | |
| Water | 24 µl | 202 µl | 1 210 µl |
| 10X KAPA A-Tailing Buffer | 3 µl | 25 µl | 151 µl |
| KAPA A-Tailing Enzyme | 3 µl | 25 µl | 151 µl |
| **Total volume of mix** | **30 µl** | **252 µl** | **1 512 µl** |
| **Other components of the final reaction:** | | | |
| End repaired DNA with beads | 0 µl | | |
| Balance of water required | 0 µl | | |
| **Total reaction volume** | **50 µl** | | |

Table 5A. Composition of the ligation reaction (standard, uninterrupted protocol).

| Component | 1 Library | 8 Libraries (incl. 5% excess) | 48 Libraries (incl. 5% excess) |
|---|---|---|---|
| **Ligation Master Mix:** | | | |
| Water | 30 µl | 252 µl | 1 512 µl |
| 5X KAPA Ligation Buffer | 10 µl | 84 µl | 504 µl |
| KAPA T4 DNA Ligase | 5 µl | 42 µl | 252 µl |
| **Total master mix volume** | **45 µl** | **378 µl** | **2 268 µl** |
| **Other components of the final reaction:** | | | |
| A-tailed DNA with beads | 0 µl | | |
| Adapter (0.7 – 60 µM, as appropriate) | 5 µl | | |
| Balance of water required | 0 µl | | |
| **Total reaction volume** | **50 µl** | | |

Table 5B. Composition of the ligation reaction if process is stopped after A-tailing.

| Component | 1 Library | 8 Libraries (incl. 5% excess) | 48 Libraries (incl. 5% excess) |
|---|---|---|---|
| **Resuspend beads in mix of:** | | | |
| Water | 16 µl | 134 µl | 806 µl |
| 5X KAPA Ligation Buffer | 4 µl | 34 µl | 202 µl |
| KAPA T4 DNA Ligase | 0 µl | 0 µl | 0 µl |
| **Total volume of mix** | **20 µl** | **168 µl** | **1 008 µl** |
| **To resume, add mix of:** | | | |
| Water | 14 µl | 118 µl | 706 µl |
| 5X KAPA Ligation Buffer | 6 µl | 50 µl | 302 µl |
| KAPA T4 DNA Ligase | 5 µl | 42 µl | 252 µl |
| **Total volume of mix** | **25 µl** | **210 µl** | **1 260 µl** |
| **Other components of the final reaction:** | | | |
| A-tailed DNA with beads | 0 µl | | |
| Adapter (0.7 – 60 µM, as appropriate) | 5 µl | | |
| Balance of water required | 0 µl | | |
| **Total reaction volume** | **50 µl** | | |

Table 6. Composition of the library amplification reaction.

| Component | 1 Library | 8 Libraries (incl. 5% excess) | 48 Libraries (incl. 5% excess) |
|---|---|---|---|
| **Library Amplification Master Mix:** | | | |
| Water | 0 µl | 0 µl | 0 µl |
| 2X KAPA HiFi HotStart ReadyMix | 25 µl | 210 µl | 1 260 µl |
| **Total master mix volume** | **25 µl** | **210 µl** | **1 260 µl** |
| **Other components of the final reaction:** | | | |
| Adapter-ligated library DNA | 20 µl | | |
| PCR Primer Premix (5 µM each primer) | 5 µl | | |
| Balance of water required | 0 µl | | |
| **Total reaction volume** | **50 µl** | | |

Table 7. Approximate volumes of additional reagents required.

| Reagent | 1 Library | 8 Libraries (incl. 5% excess) | 48 Libraries (incl. 5% excess) |
|---|---|---|---|
| **PEG/NaCl SPRI® Solution (provided in kit):** | | | |
| A-tailing cleanup | 90 µl | 756 µl | 4 536 µl |
| 1st Adapter ligation cleanup | 50 µl | 420 µl | 2 520 µl |
| 2nd Adapter ligation cleanup/Dual-SPRI® size selection (max.) | 60 µl | 504 µl | 3 024 µl |
| **Total volume required** | **200 µl** | **1.68 ml** | **~10.1 ml** |
| **AMPure® XP reagent (not supplied):** | | | |
| End repair cleanup | 120 µl | 1 008 µl | 6 048 µl |
| Dual-SPRI® size selection | ~20 µl | ~168 µl | ~1 008 µl |
| Post amplification cleanup | 50 µl | 420 µl | 2 520 µl |
| **Total volume required** | **~190 µl** | **~1.6 ml** | **~9.6 ml** |
| **80% Ethanol (freshly prepared; not supplied):** | | | |
| End repair cleanup | 400 µl | 3.36 ml | 20.2 ml |
| A-tailing cleanup | 400 µl | 3.36 ml | 20.2 ml |
| 1st Adapter ligation cleanup | 400 µl | 3.36 ml | 20.2 ml |
| 2nd Adapter ligation cleanup/Dual-SPRI® size selection | 400 µl | 3.36 ml | 20.2 ml |
| Post amplification cleanup | 400 µl | 3.36 ml | 20.2 ml |
| **Total volume required** | **2 ml** | **16.8 ml** | **101 ml** |
| **Elution buffer (10 mM Tris-HCl, pH 8.0; not supplied):** | | | |
| Adapter ligation cleanup/Dual-SPRI® size selection (max.) | 125 µl | 1 050 ml | 6 300 ml |
| Amplification cleanup | 25 µl | 210 ml | 1 260 ml |
| **Total volume required** | **150 µl** | **1 260 ml** | **7 560 ml** |

## 2. End Repair Reaction Setup

2.1 Assemble each end repair reaction as follows:

| | |
|---|---|
| Fragmented, double-stranded DNA | 50 µl |
| End Repair Master Mix (Table 3) | 20 µl |
| **Total reaction volume** | **70 µl** |

2.2 Mix, and incubate at 20 °C for 30 min. Proceed immediately to the next step.

## 3. End Repair Cleanup

3.1 To each 70 µl end repair reaction, add:

| | |
|---|---|
| Agencourt® AMPure® XP reagent | 120 µl |
| **Total volume per well/tube** | **190 µl** |

3.2 Mix thoroughly by pipetting up and down multiple times.

3.3 Incubate the plate/tube at room temperature for 5 – 15 min to allow the DNA to bind to the beads.

3.4 Place the plate/tube 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 on the magnet, add 200 µl of 80% ethanol.

3.7 Incubate the plate/tube at room temperature for ≥30 sec.

3.8 Carefully remove and discard the ethanol.

3.9 Keeping the plate/tube on the magnet, add 200 µl of 80% ethanol.

3.10 Incubate the plate/tube 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 Allow the beads to dry at room temperature, sufficiently for all the ethanol to evaporate. **Caution: over-drying the beads may result in dramatic yield loss.**

3.13 Remove the plate/tube from the magnet.

## 4. A-Tailing Reaction Setup

4.1 To each well/tube containing beads with end-repaired DNA, add:

| | |
|---|---|
| Water (if required) | 0 µl |
| A-Tailing Master Mix (Table 4A)† | 50 µl |
| **Total reaction volume** | **50 µl** |

†For a safe stopping point, resuspend the beads in 20 µl 1X A-Tailing Buffer, cover the reaction and store at 4 °C for up to 24 hours. Resume the reaction by adding the rest of the reaction components, as outlined in Table 4B (p. 10).

4.2 Thoroughly resuspend the beads by pipetting up and down multiple times.

4.3 Incubate at 30 °C for 30 min. Proceed immediately to the next step.

## 5. A-Tailing Cleanup

5.1 To each well/tube containing the 50 µl A-tailing reaction with beads, add:

| | |
|---|---|
| PEG/NaCl SPRI® Solution | 90 µl |
| **Total volume per well/tube** | **140 µl** |

5.2 Thoroughly resuspend the beads by pipetting up and down multiple times.

5.3 Incubate the plate/tube at room temperature for 5 – 15 min to allow the DNA to bind to the beads.

5.4 Place the plate/tube 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 on the magnet, add 200 µl of 80% ethanol.

5.7 Incubate the plate/tube at room temperature for ≥30 sec.

5.8 Carefully remove and discard the ethanol.

5.9 Keeping the plate/tube on the magnet, add 200 µl of 80% ethanol.

5.10 Incubate the plate/tube 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 Allow the beads to dry at room temperature, sufficiently for all the ethanol to evaporate. **Caution: over-drying the beads may result in dramatic yield loss.**

5.13 Remove the plate/tube from the magnet.

## 6. Adapter Ligation Reaction Setup

6.1 To each well/tube containing the dried beads with A-tailed DNA, add:

| | |
|---|---|
| Water (if required) | 0 µl |
| Ligation Master Mix (Table 5A)† | 45 µl |
| Adapter (concentration as required) | 5 µl |
| **Total volume per well/tube** | **50 µl** |

†For a safe stopping point, resuspend the beads in 20 µl 1X Ligation Buffer, cover the reaction and store at 4 °C for up to 24 hours. Resume the reaction by adding the rest of the reaction components, as outlined in Table 5B (p. 10).

6.2 Thoroughly resuspend the beads by pipetting up and down multiple times.

6.3 Incubate at 20 °C for 15 min. Proceed immediately to the next step.

## 7. Adapter Ligation Cleanup

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

### First Post-Ligation Cleanup

7.1 To each 50 µl ligation reaction with beads, add:

| | |
|---|---|
| PEG/NaCl SPRI® Solution | 50 µl |
| **Total volume per well/tube** | **100 µl** |

7.2 Thoroughly resuspend the beads by pipetting up and down multiple times.

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

7.4 Place the plate/tube 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 on the magnet, add 200 µl of 80% ethanol.

7.7 Incubate the plate/tube at room temperature for ≥30 sec.

7.8 Carefully remove and discard the ethanol.

7.9 Keeping the plate/tube on the magnet, add 200 µl of 80% ethanol.

7.10 Incubate the plate/tube 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 Allow the beads to dry at room temperature, sufficiently for all the ethanol to evaporate. **Caution: over-drying the beads may result in dramatic yield loss.**

7.13 Remove the plate/tube from the magnet**.**

7.14 Thoroughly resuspend the beads in an appropriate volume of elution buffer (10 mM Tris-HCl, pH 8.0). Consult **Important Parameters: Post-ligation cleanup** (p. 5). Recommended volumes are as follows:

- If proceeding to a second post-ligation cleanup, resuspend the beads in 50 µl.
- If proceeding to dual-SPRI® size selection, 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 up to 24 hours.

7.15 Incubate the plate/tube at room temperature for 2 min to allow the DNA to elute off the beads.

### Second Post-Ligation Cleanup

7.16 To the 50 µl of cleaned-up, adapter-ligated DNA with beads, add:

| | |
|---|---|
| PEG/NaCl SPRI® Solution | 50 µl |
| **Total volume** | **100 µl** |

7.17 Thoroughly resuspend the beads by pipetting up and down multiple times.

7.18 Incubate the plate/tube at room temperature for 5 – 15 min to allow the DNA to bind to the beads.

7.19 Place the plate/tube on a magnet to capture the beads. Incubate until the liquid is clear.

7.20 Carefully remove and discard the supernatant.

7.21 Keeping the plate/tube on the magnet, add 200 µl of 80% ethanol.

7.22 Incubate the plate/tube at room temperature for ≥30 sec.

7.23 Carefully remove and discard the ethanol.

7.24 Keeping the plate/tube on the magnet, add 200 µl of 80% ethanol.

7.25 Incubate the plate/tube at room temperature for ≥30 sec.

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

7.27 Allow the beads to dry at room temperature, sufficiently for all the ethanol to evaporate. **Caution: over-drying the beads may result in dramatic yield loss.**

**Second Post-Ligation Cleanup (continued)**

7.28 Remove the plate/tube from the magnet.

7.29 Thoroughly resuspend the beads in 25 µl of elution buffer (10 mM Tris-HCl, pH 8.0). Please refer to the relevant notes under **Important Parameters**. Incubate the plate/tube at room temperature for 2 min to allow the DNA to elute off the beads.

7.30 Place the plate/tube on a magnet to capture the beads. Incubate until the liquid is clear.

7.31 Transfer the clear supernatant to a new plate/tube and proceed with library amplification (Section 9), QC and/ or sequencing, as appropriate.

## 8. Dual-SPRI® Size Selection

8.1 To 100 µl of resuspended DNA with beads, add:

| | |
|---|---|
| PEG/NaCl SPRI® Solution | 60 µl |
| **Total volume per well/tube** | **160 µl** |

8.2 Thoroughly resuspend the beads by pipetting up and down multiple times.

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

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

8.5 Carefully transfer 155 µl of the supernatant(s) containing library fragments smaller than ~450 bp to a new plate/tube. **It is critical to not transfer any beads with the supernatant.**

8.6 Discard the old plate/tube with the beads carrying library fragments larger than ~450 bp.

8.7 To each 155 µl supernatant in the new plate/tube, add:

| | |
|---|---|
| Agencourt® AMPure® XP reagent | 20 µl |
| **Total volume per well/tube** | **175 µl** |

8.8 Thoroughly resuspend the beads by pipetting up and down multiple times.

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

8.10 Place the plate/tube on a magnet to capture the beads. Incubate until the liquid is clear.

8.11 Carefully remove and discard the supernatant.

8.12 Keeping the plate/tube on the magnet, add 200 µl of 80% ethanol.

8.13 Incubate the plate/tube at room temperature for ≥30 sec.

8.14 Carefully remove and discard the ethanol.

8.15 Keeping the plate/tube on the magnet, add 200 µl of 80% ethanol.

8.16 Incubate the plate/tube at room temperature for ≥30 sec.

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

8.18 Allow the beads to dry at room temperature, sufficiently for all the ethanol to evaporate. **Caution: over-drying the beads may result in dramatic yield loss.**

8.19 Remove the plate/tube from the magnet.

8.20 Thoroughly resuspend the beads in 25 µl of elution buffer (10 mM Tris-HCl, pH 8.0). Please refer to the relevant notes under **Important Parameters**. Incubate the plate/tube at room temperature for 2 min to allow the DNA to elute off the beads.

8.21 Place the plate/tube on a magnet to capture the beads. Incubate until the liquid is clear.

8.22 Transfer the clear supernatant to a new plate/tube and proceed with library amplification (Section 9), QC and/or sequencing, as appropriate.

## 9. Library Amplification Reaction Setup

Please refer to **Important Parameters: Library amplification** and Section 7 of the **KAPA NGS Library Preparation Technical Guide** for more information on optimizing library amplification.

9.1 Assemble each library amplification reaction as follows:

| | |
|---|---|
| Water (if required) | 0 µl |
| Library DNA | 20 µl |
| Library Amplification Master Mix (Table 6) | 25 µl |
| PCR Primer Premix (5 µM each primer)† | 5 µl |
| **Total volume per well/tube** | **50 µl** |

†The recommended final concentration of each primer in the library amplification reaction is 500 nM. This may be increased to a final concentration of 2 µM of each primer if necessary.

9.2 Perform PCR with the thermocycling parameters outlined on the next page.

9.3 Store the plate/tube at 4 °C or -20 °C for up to 72 hours, or proceed directly to Section 10.

## 9. Library Amplification Reaction Setup (continued)

| Step | Temp | Duration | Cycles |
|---|---|---|---|
| Initial denaturation | 98 °C | 45 sec | 1 |
| Denaturation | 98 °C | 15 sec | Minimum number required for optimal amplification (Table 2) |
| Annealing† | 60 °C | 30 sec | |
| Extension | 72 °C | 30 sec | |
| Final extension | 72 °C | 60 sec | 1 |
| Stop reaction | 4 °C | Hold | Hold |

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

## 10. Library Amplification Cleanup

10.1 To each well/tube containing 50 µl library amplification reaction, add:

| | |
|---|---|
| Agencourt® AMPure® XP reagent | 50 µl |
| **Total volume per well/tube** | **100 µl** |

10.2 Thoroughly resuspend the beads by pipetting up and down multiple times.

10.3 Incubate the plate/tube at room temperature for 5 – 15 min to allow the DNA to bind to the beads.

10.4 Place the plate/tube on a magnet to capture the beads. Incubate until the liquid is clear.

10.5 Remove and discard the supernatant.

10.6 Keeping the plate/tube on the magnet, add 200 µl of 80% ethanol.

10.7 Incubate the plate/tube at room temperature for ≥30 sec.

10.8 Remove and discard the ethanol.

10.9. Keeping the plate/tube on the magnet, add 200 µl of 80% ethanol.

10.10 Incubate the plate/tube at room temperature for ≥30 sec.

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

10.12 Allow the beads to dry at room temperature, sufficiently for all the ethanol to evaporate. **Caution: over-drying the beads may result in dramatic yield loss.**

10.13 Remove the plate/tube from the magnet.

10.14 Thoroughly resuspend the beads in an appropriate volume of elution buffer (10 mM Tris-HCl, pH 8.0). Please refer to the relevant notes under **Important Parameters**. Incubate the plate/tube at room temperature for 2 min to allow the DNA to elute off the beads.

10.15 Place the plate/tube on a magnet to capture the beads. Incubate until the liquid is clear.

10.16 Transfer the clear supernatant to a new plate/tube and proceed with library QC, target enrichment or sequencing, as appropriate.

## Appendix 1:

## Library construction guidelines for target enrichment with the Roche Nimblegen™ SeqCap EZ 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 Roche Nimblegen™ **SeqCap EZ SR User Guide** (v3.0 and 4.0) 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.

### 1. Library Construction

Prepare libraries from fragmented, human genomic DNA according to the protocol outlined on pp. 9 – 15 of this document. Specific recommendations are as follows:

- For optimal results with 2 x 100 bp paired-end sequencing, input DNA should be fragmented to an average size distribution of 180 – 220 bp, using a Covaris instrument. For 2 x 250 bp paired-end sequencing, a slightly larger insert size may be preferred. The size distribution of the fragmented DNA should be confirmed electrophoretically prior to library construction, using an PerkinElmer LabChip® GX, Agilent Bioanalyzer or TapeStation, or similar instrument.
- The input of fragmented, dsDNA into the end repair reaction should be in the range of 100 ng – 1 µg.
- The amount of adapter should be varied according to the amount of input DNA. Please refer to **Important Parameters: Adapter design and concentration** (p. 4) in this regard. When using Illumina® TruSeq™ adapter 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) 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) and use 5 µl per 50 µl ligation reaction.
- After adapter ligation, Roche typically recommends a dual-SPRI® size selection. This is achieved by performing the First Post-Ligation Cleanup (Steps 7.1 – 7.15), eluting the DNA in 100 µl elution buffer (10 mM Tris-HCl, pH 8.0), and proceeding to the Dual-SPRI® Size Selection (Section 8). The dual-SPRI® size selection is designed to eliminate adapter-ligated library fragments >450 bp and <250 bp. Please note that dual-SPRI® size selection can result 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 SPRI® cleanups (Steps 7.1 – 7.31) 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 input DNA is limiting.
- Irrespective of whether the "No Size Selection" (two consecutive 1X SPRI® cleanups) or Dual-SPRI® Size Selection strategy is followed, thoroughly resuspend the final, dried beads with cleaned/size-selected, adapter-ligated library in 25 µl elution buffer. Proceed directly to Section 2 below.

### 2. Pre-Capture LM-PCR

2.1 Assemble each pre-capture LM-PCR in a PCR tube or plate as follows:

| | |
|---|---|
| 2X KAPA HiFi HotStart ReadyMix | 25 µl |
| 5 µM TS-PCR Oligo 1 | 2.5 µl |
| 5 µM TS-PCR Oligo 2 | 2.5 µl |
| **Total volume per well/tube** | **30 µl** |

2.2 Place the plate/tube with beads resuspended in elution buffer (from Section 1) on a magnet to capture the beads. Incubate until the liquid is clear.

2.3 Transfer 20 µl of the clear supernatant (or PCR-grade water for negative controls) to the appropriate PCR tube or well with pre-capture LM-PCR mix. Mix thoroughly by pipetting up and down.

2.4 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 | 7 - 9† |
| Annealing | 60 °C | 30 sec | |
| Extension | 72 °C | 30 sec | |
| Final extension | 72 °C | 60 sec | 1 |
| Stop reaction | 4 °C | Hold | Hold |

†7 cycles are recommended for 1 µg input DNA. For lower input, or FFPE DNA, one or two additional cycles may be needed to produce sufficient material for capture.

2.5 Store the plate/tube at 4 °C for up to 72 hours or proceed directly to Section 3.

**Please note:**

- The sequences of the primers used for pre-capture LM-PCR may be found in the Roche Nimblegen™ **SeqCap EZ SR User Guide** v3.0.

## 2. Pre-Capture LM-PCR (notes, continued)

- The final concentration of each primer in the pre-capture LM-PCR is 250 nM. This should be increased to 500 nM when 1 µg input DNA is used for library construction, or if electrophoretic analysis of pre-capture LM-PCR products suggests that over-amplification is occurring. The best way to increase the final primer concentration is to increase the concentration of each primer stock from 5 to 10 µM, and use 2.5 µl of each primer.
- The forward and reverse primers may be supplied in a premix containing **each** of the primers at a final concentration of 2.5 µM (for a final concentration of 250 nM of each primer), or 5 µM (for a final concentration of 500 nM of each primer). If such a primer premix is used, the pre-capture LM-PCR mix will consist of 25 µl of KAPA HiFi HotStart ReadyMix (2X) and 5 µl of the primer premix.

## 3. Cleanup and QC of the Pre-Capture LM-PCR Amplified Sample Library

3.1 Amplified libraries can be purified using Agencourt® AMPure® XP reagent or the QIAGEN® QIAquick® PCR Purification Kit.

When using Agencourt® AMPure® XP reagent, follow the instructions in Section 10 (p. 15), with the following exceptions:

- Increase the volume of AMPure® XP reagent from 50 µl to 90 µl, for a 1.8X cleanup.
- Elute the cleaned library in 50 µl **PCR-grade water** instead of elution buffer.

When using the QIAGEN® QIAquick® PCR Purification Kit, follow the manufacturer's instructions, with the following exceptions:

- Elute the cleaned library in 50 µl **PCR-grade water** instead of Buffer EB.

3.2. Quantify the amplified library with the KAPA Library Quantification Kit, or with a NanoDrop™ spectrophotometer, and analyze 1 µl of the sample using an Agilent 2100 Bioanalyzer DNA 1000 Kit. For optimal results, the yield of amplified library should be >1 µg, and the library fragment size distribution should be 150 bp – 500 bp.

## 4. Hybridization of the Amplified Sample Library to the SeqCap EZ Library

4.1 Set up hybridization reactions according to the instructions in **Chapter 5. Hybridizing the Sample and SeqCap EZ Libraries** of the **SeqCap EZ SR User Guide** (v4.0).

## 5. Washing and Recovery of the Captured DNA Sample

5.1 Follow the instructions in **Chapter 6. Washing and Recovering Captured Multiplex DNA Sample** of the **SeqCap EZ SR User Guide** (v4.0) for binding of the captured DNA sample to streptavidin beads and washing of the bead-bound DNA.

## 6. 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.

Notes given at the end of Step 2.5 of the section on pre-capture LM-PCR also apply to post-capture LM-PCR.

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

| | |
|---|---|
| 2X KAPA HiFi HotStart ReadyMix | 25 µl |
| 5 µM TS-PCR Oligo 1 | 2.5 µl |
| 5 µM TS-PCR Oligo 2 | 2.5 µl |
| **Total volume per well/tube** | **30 µl** |

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

6.3 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 -20 °C.

6.4 Perform the post-capture LM-PCR with the following thermocycling parameters:

| 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 | 60 sec | 1 |
| Stop reaction | 4 °C | Hold | Hold |

## 6. Post-Capture LM-PCR (continued)

6.5 Store the plate/tube for up to 72 hours at 4 °C or proceed directly to Section 7.

## 7. Cleanup and QC of the Post-Capture LM-PCR Amplified Sample Library

7.1 Amplified libraries can be purified using Agencourt® AMPure® XP reagent or the QIAGEN® QIAquick® PCR Purification Kit.

When using Agencourt® AMPure® XP reagent, transfer the supernatants of each set of two PCRs from the streptavidin beads, and pool these into a single 1.5 ml microtube or prior to cleanup. Follow the instructions in Section 10 (p. 15), with the following exception:

- Increase the volume of AMPure® XP reagent to 216 µl, for a 1.8X cleanup.

Alternatively, the supernatants of the two corresponding PCRs may be cleaned up separately, and the DNA pooled after elution.

When using the QIAGEN® QIAquick® PCR Purification Kit, transfer the supernatants of each set of two PCRs from the streptavidin beads, and pool these into a single tube for purification using a single column. Follow the manufacturer's instructions and elute the final library in the appropriate volume of Buffer EB.

7.2 Quantify the final, amplified library with the KAPA Library Quantification Kit, or with 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 size distribution should be 150 bp – 500 bp.

## 8. Determine the Sample Enrichment using qPCR

8.1 Follow the instructions in **Chapter 8. Measuring Enrichment Using qPCR** of the **SeqCap EZ SR User Guide** (v4.0) for estimating the relative fold enrichment achieved for the amplified, captured DNA sample.

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