KAPABIOSYSTEMS

Technical Data Sheet

KAPA Hyper Prep Kit
Illumina® platforms

KR0961 – v1.14

This Technical Data Sheet provides product information and a detailed protocol for the KAPA Hyper Prep Kit (Illumina® platforms).

Contents

Kit Codes and Components

KK8500 8 libraries	KAPA End Repair & A-Tailing Buffer KAPA End Repair & A-Tailing Enzyme KAPA Ligation Buffer KAPA DNA Ligase KAPA HiFi HotStart ReadyMix KAPA Library Amplification Primer Mix	60 µl 25 µl 245 µl 80 µl 250 µl 50 µl
KK8502 24 libraries	KAPA End Repair & A-Tailing Buffer KAPA End Repair & A-Tailing Enzyme KAPA Ligation Buffer KAPA DNA Ligase KAPA HiFi HotStart ReadyMix KAPA Library Amplification Primer Mix	170 µl 72 µl 720 µl 240 µl 690 µl 138 µl
KK8504 96 libraries	KAPA End Repair & A-Tailing Buffer KAPA End Repair & A-Tailing Enzyme KAPA Ligation Buffer KAPA DNA Ligase KAPA HiFi HotStart ReadyMix KAPA Library Amplification Primer Mix	680 µl 290 µl 2.88 ml 960 µl 3 ml 600 µl

For PCR-free workflows, **KK8501 (8 libraries)**, **KK8503 (24 libraries)** and **KK8505 (96 libraries)** are also available. These kits do not include KAPA HiFi HotStart ReadyMix or KAPA Library Amplification Primer Mix.

Quick Notes

- This kit is used for the construction of DNA libraries for Illumina® sequencing in 2 – 3 hours, with minimal hands-on time.
- The novel one-tube chemistry improves library yield and quality for FFPE and low-input libraries.
- Kits contain KAPA HiFi HotStart ReadyMix and an optimized Library Amplification Primer Mix for high-efficiency, low-bias library amplification.
- Kits without an amplification module are available for PCR-free workflows.
- The process workflow (p. 7) provides an overview of the library construction process. The appendix provides protocols for dual-SPRI® size selection.
- Separate, concentrated enzyme formulations and reaction buffers for end repair and A-tailing as well as ligation provide the best combination of product stability, convenience, and efficiency.
- Adapters are not supplied with this kit, but can be obtained from any reputable oligonucleotide vendor.
- SPRI® beads required for post-ligation and post-amplification cleanups are not included in the kit.

Product Description

The KAPA Hyper Prep Kit is designed for the rapid construction of libraries for Illumina® sequencing from fragmented, double-stranded DNA (dsDNA).

This product is derived from Kapa's existing HTP/LTP Library Preparation Kit for Illumina® platforms, which was carefully optimized for the construction of high-quality libraries from a wide range of DNA inputs and sample types (including FFPE and cell-free DNA), for various sequencing applications. In the KAPA Hyper Prep Kit, a streamlined protocol is used, combining enzymatic steps and reducing the number of SPRI® bead cleanups, without compromising library yield or quality. The streamlined protocol reduces hands-on and overall library preparation time, and improves consistency by reducing the number of sample handling steps.

The kit contains all of the enzymes and reaction buffers required for the following steps of library construction:

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

The kit provides a single, concentrated buffer and a single enzyme mixture for each of the two library construction steps, but does not include adapters or SPRI® beads required for cleanups after adapter ligation and library amplification. Kits without amplification reagents (KK8501, KK8503 and KK8505) are available for PCR-free workflows.

In order to maximize sequence coverage uniformity, it is critical to minimize library amplification bias. KAPA HiFi DNA Polymerase is designed for low-bias, high-fidelity PCR, and is the reagent of choice for NGS library amplification.[1,2,3,4] The KAPA Hyper Prep Kit includes KAPA HiFi HotStart ReadyMix (2X), a ready-to-use PCR mix comprising all the components for library amplification, except primers and template. Kits also include KAPA Library Amplification Primer Mix, which is compatible with all libraries prepared with full-length Illumina® adapters. The kit can 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. Oyola, S.O. *et al. BMC Genomics* **13**, 1 (2012).
2. Quail M.A. *et al. Nature Methods* **9**, 10 (2012).
3. Quail M.A. *et al. BMC Genomics* **13**: 341 (2012).
4. Ross, M.G. *et al. Genome Biology* **14**: R51 (2013).

Product Applications

The KAPA Hyper Prep Kit is ideally suited for low- and high-throughput NGS library construction workflows that require 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 KAPA HiFi HotStart Uracil+ ReadyMix for library amplification)

Product Specifications

Shipping and storage

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

Handling

Always ensure that 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) may not freeze completely, even when stored at -15 °C to -25 °C. Nevertheless, always ensure that the KAPA HiFi HotStart ReadyMix is fully thawed 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 generic, and reaction parameters may be adjusted as required to optimize performance, efficiency, and cost-effectiveness. Several critical parameters are discussed in this section.

Safe stopping points

The library construction process, from end repair and A-tailing to final, amplified library, can be performed in 2 – 3 hours, depending on experience and the number of samples being processed. If necessary, the protocol may be paused safely after completion of the post-ligation cleanup (Step 3.17), or prior to the post-amplification cleanup (Step 4.4).

Adapter-ligated library DNA may 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. However, please note that libraries constructed for target enrichment must be eluted and stored in PCR-grade water.

Paramagnetic SPRI® beads and reaction cleanups

- 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 use.
- The incubation times provided for reaction cleanups are guidelines only, and may be modified/optimized according to current protocols, previous experience, and specific equipment and samples in order to maximize library construction efficiency and throughput.
- The time required for complete capture of magnetic beads varies according to the reaction vessel and magnet used. It is important not to discard or transfer any beads with the removal or transfer of supernatant. Capture times should be optimized accordingly.
- The volumes of 80% ethanol used for bead washes may be adjusted to accommodate smaller reaction vessels and/or limited pipetting capacity, but it is important that the beads are entirely submerged during the wash steps. Where possible, use a wash volume that is equal to the volume of sample plus AMPure® XP reagent.
- 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 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. However, libraries constructed for target enrichment must be eluted and stored in PCR-grade water to allow for drying of DNA prior to target enrichment.

Input DNA and fragmentation

- This protocol has been validated for library construction from 1 ng – 1 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.
- The above refers to the end repair and A-tailing reaction input. 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.
- Solutions containing high concentrations of EDTA and strong buffers may negatively affect the end repair and A-tailing 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.
- The proportion of fragmented DNA that is successfully converted to adapter-ligated molecules decreases as input is reduced. When constructing libraries from >10 ng fragmented genomic DNA, 5 – 40% of input DNA is typically recovered as adapter-ligated molecules. Workflows with additional SPRI® cleanups or size selection prior to adapter ligation are likely to result in a lower yield of adapter-ligated molecules.

Adapter design and concentration

- This protocol has been validated using indexed Illumina® TruSeq™, Roche Nimblegen™ SeqCap EZ and Agilent SureSelect^{XT2} adapters, but the kit is also compatible with other adapters of similar design.
- Adapter concentration affects ligation efficiency, as well as adapter and adapter-dimer carry-over through the post-ligation cleanup. The optimal adapter concentration for your workflow represents a compromise between cost and the above factors.

Adapter design and concentration (continued)

- Ligation efficiency is robust for adapter:insert molar ratios ranging from 10:1 to >100:1. Please refer to Table 1 for the recommended adapter concentrations for a range of input DNA amounts.
- High adapter:insert molar ratios (>100:1) are beneficial for low-input applications. When optimizing workflows for DNA inputs ≤25 ng, we recommended evaluating two or three adapter concentrations. Try the recommended adapter concentration (Table 1), as well as one or two additional concentrations in the range of 2 – 10 times higher.
- To accommodate different adapter concentrations within a batch of samples processed together, it is best 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 not recommended.

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

Input DNA	Adapter stock concentration	Final adapter concentration
50 ng – 1 µg	15 µM	680 nM
25 ng	7.5 µM	340 nM
10 ng	3 µM	136 µM
5 ng	1.5 µM	68 nM
2.5 ng	750 nM	34 nM
1 ng	300 nM	14 nM

Post-ligation cleanup

- It is important to remove excess unligated adapter and adapter-dimers from the library prior to library amplification or cluster generation.
- The KAPA Hyper Prep Kit chemistry reduces adapter-dimer formation, and enables efficient elimination of unused adapter and adapter-dimer with a single post-ligation cleanup. The optimal SPRI® ratio for libraries prepared from fragmented dsDNA with an average size in the range of 150 – 350 bp is 0.8X.
- The volume in which washed beads are resuspended after the post-ligation cleanup should be adjusted to suit your chosen workflow. If proceeding directly to library amplification, determine an appropriate final volume in which to elute the library DNA, keeping in mind that you may wish to divert and/or reserve some of this library material for archiving and/or QC purposes. Since an optimized 50 µl library amplification reaction can accommodate a maximum of 20 µl template DNA, an elution volume of ~25 µl is recommended.

Size selection

- If required, any commonly used size selection technique (e.g. dual-SPRI® or an electrophoresis-based method) may be integrated in this protocol.
- Size selection may be carried out at several points in the overall workflow, for example:
  - prior to end repair of fragmented DNA.
  - after the post-ligation cleanup.
  - after library amplification.

  The standard KAPA Hyper Prep protocol (pp. 8 – 9) does not include size selection. Please refer to the **Appendix** for detailed dual-SPRI® size selection protocols.
- 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 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 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 typically results in the observation of secondary, higher molecular weight peaks when amplified libraries are analyzed electrophoretically. These higher molecular weight peaks are artifacts, 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 **Library amplification** subsection for more information. Note that for some workflows, post-amplification size selection may be preferred (e.g. ChIP-seq library construction).
- 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. If size selection is performed after ligation, it is important to perform at least one SPRI® bead cleanup prior to performing bead- or electrophoresis-based size selection.

Library amplification

- 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 2.8×10^{-7} errors/base, equivalent to 1 error in 3.5×10^{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.
- KAPA Hyper Prep Kits contain KAPA Library Amplification Primer Mix, designed to eliminate or delay primer depletion during library amplification reactions performed with KAPA HiFi HotStart ReadyMix. These primers are based on the P5 and P7 Illumina® flow cell sequences, and are suitable for the amplification of libraries prepared with full-length adapters. User-supplied primer mixes may be used in combination with incomplete or custom adapters. Please contact **support@kapabiosystems.com** for guidelines on the formulation of user-supplied library amplification primers.
- 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 enrichment or sequencing).
- If cycled to completion (**not recommended**) a single 50 µl library amplification PCR, performed as described in Section 4, can produce 8 – 10 µg of amplified library. To minimize over-amplification and associated undesired artifacts, the number of amplification cycles should be tailored to produce the optimal amount of amplified library required for downstream processes. This is typically in the range of 250 ng – 1.5 µg of final, amplified library. Table 2 provides recommended cycle numbers for libraries prepared from high quality input DNA to obtain ~100 ng or ~1 µg of amplified library.

Table 2. Recommended cycle numbers to generate 100 ng or 1 µg of amplified DNA from 1 ng – 1 µg of input DNA.

Input DNA[1]	Number of cycles required to generate:	
	100 ng	1 µg
1 µg	0 – 1	2 – 3
500 ng	0 – 1	3 – 4
250 ng	1 – 3	4 – 6
100 ng	3 – 4	6 – 7
50 ng	4 – 5	7 – 8
25 ng	5 – 7	8 – 10
10 ng	8 – 11	11 – 14
5 ng	10 – 13	13 – 16
2.5 ng	12 – 14	15 – 17
1 ng	14 – 16	17 – 19

[1] Input into end repair and A-tailing reaction

- Quantification of adapter-ligated libraries prior to amplification can facilitate optimization, particularly when a library construction workflow is first established or optimized. The amount of template DNA (adapter-ligated molecules) available for library amplification may be determined using the KAPA Library Quantification Kit. Using a simple calculation for exponential amplification, it is then possible to determine the number of PCR cycles required to achieve the desired quantity of amplified library. A calculator for this purpose is available upon request from **support@kapabiosystems.com**. Refer to Table 3 for the number of cycles recommended to obtain 1 µg of DNA from 0.5 ng – 500 ng of adapter-ligated DNA. Please note that the **actual** optimal number of amplification cycles may be 1 – 3 cycles higher or lower, depending on the sample type and size distribution of the input DNA.

Library amplification (continued)

- 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 the adapters used are complete, and will support sample indexing (where required), cluster amplification, and sequencing. Omitting library amplification further streamlines the workflow and reduces the overall library preparation time to <2 hours. The high conversion efficiency achievable with the KAPA Hyper Prep Kit enables PCR-free workflows from as little as 100 ng input DNA. KAPA Hyper Prep Kits without amplification reagents (KK8501, KK8503 and KK8505) are available for PCR-free workflows.

Table 3. Theoretical number of cycles required to obtain ~1 µg of amplified library DNA from 0.5 – 500 ng of adapter-ligated library DNA†.

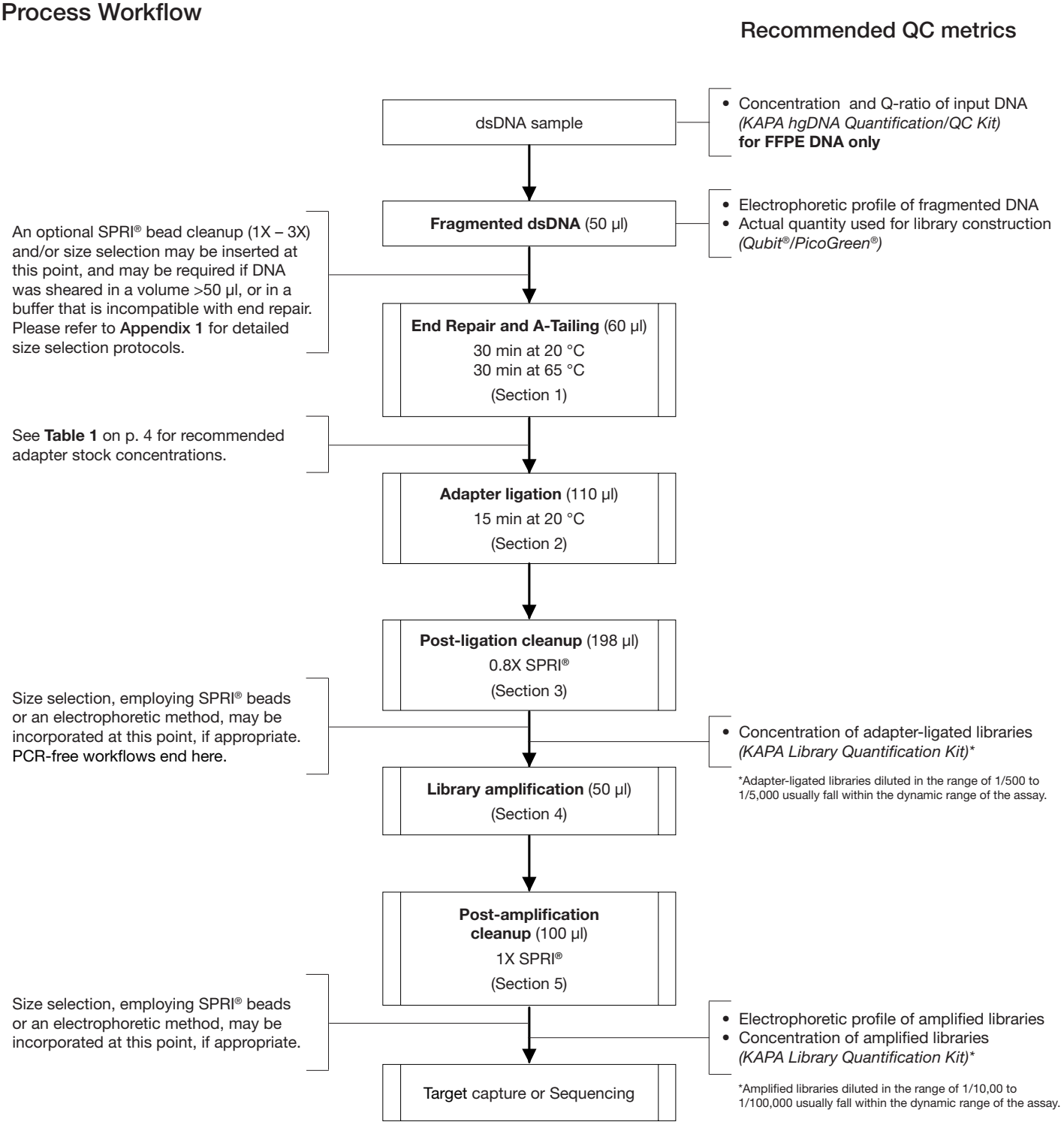
Adapter-ligated library DNA	Number of cycles require to generate 1 µg of library DNA
500 ng	1 – 2
100 ng	3 – 4
50 ng	5 – 6
10 ng	7 – 8
5 ng	8 – 9
1 ng	11 – 12
500 pg	12 – 13

† Guidelines are based on library concentrations determined with the qPCR-based KAPA Library Quantification Kit, and amplification with the KAPA Library Amplification Primer Mix.

Evaluating the success of library construction

- Your specific library construction workflow should be tailored and optimized to yield a sufficient amount of adapter-ligated molecules of the desired size distribution for the next step in the process (e.g. target capture or sequencing), as well as for library QC and archiving purposes.
- The size distribution of 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 any libraries that are ready for cluster 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 after adapter ligation or library amplification, 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 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 as compared to the corresponding amplified libraries. 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

Note: This protocol does not include size selection. Please refer to the **Appendix** for detailed dual-SPRI® size selection protocols.

1. End Repair and A-Tailing

1.1 Assemble each End Repair & A-Tailing reaction as follows in a tube or well of a PCR plate:

Component	Volume
Fragmented, double-stranded DNA	50 µl
End Repair & A-Tailing Buffer†	7 µl
End Repair & A-Tailing Enzyme Mix†	3 µl
Total volume	**60 µl**

† The buffer and enzyme mix may be pre-mixed and added in a single pipetting step. Premixes are stable for ≤24 hours at room temperature, for ≤1 week at 4 °C, and for ≤3 months at -20 °C.

1.2 Mix thoroughly and centrifuge briefly.

1.3 Incubate in a thermocycler with the following thermal profile:

Step	Temp	Time
End Repair & A-Tailing	20 °C	30 min
	65 °C	30 min
HOLD	4 °C	∞

1.4 Proceed immediately to the next step.

2. Adapter Ligation

2.1 Dilute adapter stocks to the appropriate concentration, as outlined in Table 1 on p. 4.

2.2 Assemble each Adapter Ligation reaction as follows:

Component	Volume
End Repair & A-Tailing reaction product	60 µl
PCR-grade water†	5 µl
Ligation Buffer†	30 µl
DNA Ligase†	10 µl
Adapter stock	5 µl
Total volume	**110 µl**

†The water, buffer and ligase enzyme may be premixed and added in a single pipetting step. Premixes are stable for ≤24 hours at room temperature, for ≤1 week at 4 °C, and for ≤3 months at -20 °C.

2.2 Mix thoroughly and centrifuge briefly.

2.3 Incubate at 20 °C for 15 min.

2.4 Proceed immediately to the next step.

3. Post-ligation Cleanup

3.1 Perform a 0.8X SPRI® cleanup by combining the following:

Component	Volume
Adapter Ligation reaction product	110 µl
Agencourt® AMPure® XP reagent	88 µl
Total volume	**198 µl**

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

3.3 Incubate the plate/tube at room temperature for 5 – 15 min to bind DNA 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 Dry the beads at room temperature, until all of the ethanol has evaporated. **Caution: over-drying the beads may result in dramatic yield loss.**

3.13 Remove the plate/tube from the magnet.

3.14 Resuspend the beads:

- To proceed with library amplification (Section 4), resuspend the beads in 25 µl of elution buffer (10 mM Tris-HCl, pH 8.0).
- To proceed with dual-SPRI® size selection (Appendix), resuspend the beads in 55 µl of elution buffer (10 mM Tris-HCl, pH 8.0).

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

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

3.17 Transfer the clear supernatant to a new plate/tube:

- To proceed with library amplification (Section 4), transfer 20 µl of supernatant.
- To proceed with dual-SPRI® size selection (Appendix), transfer 50 µl of supernatant.

4. Library Amplification

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.

4.1 Assemble each library amplification reaction as follows:

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

4.2 Mix thoroughly and centrifuge briefly.

4.3 Amplify using the following cycling protocol:

Step	Temp	Duration	Cycles
Initial denaturation	98 °C	45 sec	1
Denaturation	98 °C	15 sec	Minimum number required for optimal amplification (Table 2 or 3)
Annealing†	60 °C	30 sec	
Extension	72 °C	30 sec	
Final extension	72 °C	1 min	1
HOLD	4 °C	∞	1

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

4.4 Store the tube/plate at 4 °C or -20 °C for up to 72 hours, or proceed directly to Step 5: Post-amplification Cleanup.

5. Post-amplification Cleanup

5.1 Perform a 1X SPRI® cleanup by combining the following:

Component	Volume
Library Amplification reaction product	50 µl
Agencourt® AMPure® XP reagent	50 µl
Total volume	**100 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 bind DNA to the beads.

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

5.5 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 Dry the beads at room temperature, until all of the ethanol has evaporated. **Caution: over-drying the beads may result in dramatic yield loss.**

5.13 Remove the plate/tube from the magnet.

5.14 Thoroughly resuspend the beads in an appropriate volume of elution buffer (10 mM Tris-HCl, pH 8.0) or PCR-grade water. **Always use PCR-grade water if proceeding to target capture.**

If proceeding with dual-SPRI® size selection (Appendix), resuspend the beads in 55 µl of elution buffer.

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

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

5.17 Transfer the clear supernatant to a new plate/tube and proceed with size selection (refer to **Appendix**), library QC, target capture or sequencing, as appropriate. Store purified, amplified libraries at 4 °C (up to 1 week), or at -20 °C.

Appendix:

Dual-SPRI® size selection

Any commonly used size selection technique (e.g. dual-SPRI® or an electrophoresis-based method) may be integrated into the KAPA Hyper Prep library construction protocol. Size selection may be carried out at several points in the workflow, for example:

- prior to end repair & A-tailing of fragmented DNA.
- after post-ligation cleanup.
- after library amplification.

Whether or not size selection is performed, which size selection technique is used, and at what stage of the library construction process size selection is performed, depends on the nature of the sample, the amount of DNA available for library construction, and the sequencing application and read length. For more information on size selection, refer to **Important Parameters: Size selection**.

The dual-SPRI® protocols outlined in this Appendix are designed for the selection of **library inserts** of 150 – 350 bp in size. To obtain a larger or smaller average size, protocols may be modified as follows:

Upper size limit	Modification	Lower size limit	Modification
Increase	Decrease First Cut ratio	Increase	Decrease Second Cut ratio[1]
Decrease	Increase First Cut ratio	Decrease	Increase Second Cut ratio

[1] The second size cut should be performed with at least 0.2 volumes of AMPure® XP reagent. DNA recovery is dramatically reduced if the difference between first and second cuts is less than ~0.2 volumes. To increase the amount of DNA recovered, use more than 0.2 volumes of AMPure® XP reagent for the second cut, but note that this may result in the recovery of smaller library fragments and a broader size distribution.

The protocol provided here should effectively size select adapter-ligated library DNA with an average library insert size of 150 – 350 bp, but please note that the long single-stranded arms of Y-adapters ligated to library fragments affect size-dependent binding to SPRI® beads, as well as the perceived fragment size determined using an electrophoretic method. For this reason, the dual-SPRI® size selection parameters for adapter-ligated libraries may have to be optimized to select for the appropriate size range. For more information on dual-SPRI® size selection, refer to the **KAPA NGS Library Preparation Technical Guide**, or contact **support@kapabiosystems.com**.

Dual-SPRI® size selection protocol

A.1 Perform the first size cut (to exclude fragments corresponding to library inserts larger than ~350 bp) by adding the appropriate volume of Agencourt® AMPure® XP reagent to the DNA, as follows:

Component	Fragmented dsDNA in 50 µl (0.7X – 0.9X)	Fragmented dsDNA in 130 µl (0.7X – 0.9X)	Adapter-ligated or amplified DNA (0.6X – 0.8X)[1]
DNA to be size-selected	50 µl	130 µl	50 µl
Agencourt® AMPure® XP reagent	35 µl	91 µl	30 µl
Total volume per well/tube	**85 µl**	**221 µl**	**80 µl**

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

A.2 Thoroughly resuspend the beads by pipetting up and down multiple times.

A.3 Incubate the plate/tube at room temperature for 5 – 15 min to bind DNA fragments larger than ~350 bp to the beads.

A.4 Place the plate/tube on a magnet to capture the beads. Incubate until the liquid is clear.

A.5 Carefully transfer the required volume of supernatant containing DNA fragments smaller than ~350 bp to a new plate/tube (see table in step A6). It is critical that no beads are transferred with the supernatant. Discard the plate/tube with beads, to which DNA fragments larger than ~350 bp are bound.

Dual-SPRI® size selection protocol (continued)

A6. Perform the second size cut (to retain fragments corresponding to library inserts larger than ~150 bp) by adding 0.2 volumes of Agencourt® AMPure® XP reagent to the first size cut supernatant, as follows:

Component	Fragmented dsDNA in 50 µl (0.7X – 0.9X)	Fragmented dsDNA in 130 µl (0.7X – 0.9X)	Adapter-ligated or amplified DNA (0.6X – 0.8X)
Supernatant from first size cut	80 µl	216 µl	75 µl
Agencourt® AMPure® XP reagent	10 µl	26 µl	10 µl
Total volume per well/tube	**90 µl**	**242 µl**	**85 µl**

A.6 Thoroughly resuspend the beads by pipetting up and down multiple times.

A.7 Incubate the plate/tube at room temperature for 5 – 15 min to bind DNA fragments larger than ~150 bp to the beads.

A.8 Place the plate/tube on a magnet to capture the beads. Incubate until the liquid is clear.

A.9 Carefully remove and discard the supernatant, which contains DNA fragments smaller than ~150 bp.

A.10 Keeping the plate/tube on the magnet, add 200 µl of 80% ethanol.

A.11 Incubate the plate/tube at room temperature for ≥30 sec.

A.12 Carefully remove and discard the ethanol.

A.13 Keeping the plate/tube on the magnet, add 200 µl of 80% ethanol.

A.14 Incubate the plate/tube at room temperature for ≥30 sec.

A.15 Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.

A.16 Dry the beads at room temperature, until all of the ethanol has evaporated. **Caution: over-drying the beads may result in dramatic yield loss.**

A.17 Remove the plate/tube from the magnet.

A.18 Thoroughly resuspend the beads in the required volume of elution buffer (or water for capture libraries).

- For fragmented dsDNA, elute in ~55 µl and use 50 µl in the end repair & A-tailing reaction.
- For adapter-ligated library DNA, either elute in ~25 µl and use 20 µl in the library amplification reaction, or elute in the volume required for the next step in your workflow, e.g. target capture or sequencing.
- For amplified library DNA, elute in the volume required for the next step in your workflow, e.g. target capture or sequencing.

A.19 Incubate the plate/tube at room temperature for 2 min to elute DNA off the beads.

A.20 Place the plate/tube on a magnet to capture the beads. Incubate until the liquid is clear.

A.21 Transfer size-selected DNA to a new plate/tube and proceed with the next step in your workflow.

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