KAPABIOSYSTEMS

Technical Data Sheet

KAPA Library Preparation Kit

Ion Torrent™ Platforms

KR0573 – v2.16

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

Contents

Kapa/*Roche* Kit Codes and Components		
KK8300* *07961952001* **KK8310*** *07961987001* 8 libraries	End Repair Buffer (10X)	62 µL
	End Repair Enzyme Mix	44 µL
	Ligation and Nick Repair Buffer (5X)	123 µL
	Ligation and Nick Repair Enzyme	53 µL
	KAPA HiFi HotStart ReadyMix (2X)*	220 µL
	Library Amplification Primer Mix (10X)*	44 µL
KK8301 *07961979001* *KK8311* *07961995001* 48 libraries	End Repair Buffer (10X)	413 µL
	End Repair Enzyme Mix	295 µL
	Ligation and Nick Repair Buffer (5X)	827 µL
	Ligation and Nick Repair Enzyme	354 µL
	KAPA HiFi HotStart ReadyMix (2X)*	1480 µL
	Library Amplification Primer Mix (10X)*	295 µL

*KK8310 and KK8311 are available for PCR-free workflows and do not contain library amplification reagents.

Quick Notes

- The protocol provided in this document is generic, and may require additional tailoring and optimization depending on the intended application.
- Separate, concentrated enzyme formulations and reaction buffers provide the best combination of product stability, convenience, and efficiency.
- Generous reagent volumes are provided in the 48-reaction kits to accommodate dead volumes required for automated liquid handling platforms.
- This kit contains all the reagents needed for library construction, except for adapters and beads. KAPA Pure Beads (KK8000, KK8001, KK8002) and Adapters (barcoded and non-barcoded) are sold separately (KK8330, KK8331, KK8332, KK8333).

Product Description

This KAPA Library Preparation Kit is designed for the preparation of libraries for sequencing on the Ion Personal Genome Machine™ (PGM) and Ion Proton™ semiconductor sequencers. The kit provides all of the enzymes and reaction buffers required for constructing libraries from 100 ng – 1 µg of fragmented, double-stranded DNA via the following steps:

1. end repair: produce blunt-ended, 5'-phosphorylated fragments.
2. adapter ligation and nick repair: ligate dsDNA adapters to blunt-ended library fragments and perform nick repair to yield library fragments flanked by adapters.
3. library amplification (optional): perform PCR to amplify library fragments carrying appropriate adapter sequences on both ends.

The kit provides all of the enzymes and buffers required for library construction and amplification, but does not include adapters or beads. Enzyme formulations and reaction buffers for end repair and ligation and nick repair are supplied in convenient, concentrated formats. KAPA Pure Beads (KK8000, KK8001, KK8002) and adapters are available separately (KK8330, KK8331, KK8332, KK8333). These formulations ensure maximum stability of the reagents and high reaction efficiencies while simplifying reaction setup.

In order to maximize sequence coverage uniformity, it is critical that library amplification bias be kept to a minimum. KAPA HiFi DNA Polymerase has been designed for low-bias, high-fidelity PCR, and is the reagent of choice for next generation sequencing (NGS) library amplification.[1,2,3] The KAPA Library Preparation Kit for Ion Torrent (KK8300, KK8301) includes KAPA HiFi HotStart ReadyMix (2X) and an adapter-specific primer mix for library amplification. Kits without library amplification components (KK8310 and KK8311) are available for PCR-free workflows.

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).

Product Applications

The KAPA Library Preparation Kit for Ion Torrent Platforms is ideally suited for NGS library construction workflows that involve end repair, adapter ligation and nick repair, and library amplification (optional). The protocol may be adapted for incorporation into workflows for a wide range of NGS applications, including:

- targeted sequencing by solution hybrid selection,
- amplicon sequencing,
- whole-genome shotgun sequencing,
- RNA-seq, and
- ChIP-seq.

Product Specifications

Shipping and Storage

The enzymes supplied 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 destination country. Upon receipt, immediately store enzymes, reaction buffers, components, and primers 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. KAPA HiFi HotStart ReadyMix (2X) contains isostabilizers and may not freeze solidly, 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. Keep all enzyme components and master mixes on ice as long as possible during handling and preparation.

Quality Control

All kit components are subjected to stringent functional quality control, are free of detectable contaminating exo– and endonuclease activities, and meet strict requirements with respect to DNA contamination. Please contact Technical Support at **kapabiosystems.com/support** for more information.

Important Parameters

Master Mixes

- This kit and protocol is designed to be compatible with both low-throughput, manual library construction and high-throughput, automated workflows. For this reason, and for ease of use, components are combined into master mixes, rather than dispensed separately into individual reactions.
- Master mixes for end repair as well as ligation and nick repair, prepared as recommended in Tables 3 and 4 of this protocol, are stable for ≤7 days at -20°C, and overnight at 4°C.

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 for use with either KAPA Pure Beads (KK8000, KK8001, KK8002), or Agencourt® AMPure® XP (Beckman Coulter®). Solutions and conditions for DNA binding and size selection may differ if other beads are used.
- Observe all the storage and handling recommendations for KAPA Pure Beads or AMPure XP.
- Beads will settle gradually; always ensure that they are fully resuspended before aspirating.

  To ensure optimal DNA recovery, it is critical that DNA and the KAPA Pure Beads solution are thoroughly mixed (by vortexing or extensive up-and-down pipetting) before the DNA binding incubation.
- The incubation times provided in the protocol for reaction cleanups and size selection are provided as 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 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 the supernatant. Capture times should be optimized accordingly.
- The volumes of 80% ethanol used for the 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 wash steps.
- It is important to remove all ethanol before proceeding with subsequent reactions. However, over-drying of the beads may make them difficult to resuspend and may result in a dramatic loss of DNA. Drying of beads at room temperature for 3 – 5 min should be sufficient. ***Drying of beads at 37°C is not recommended.***
- Where appropriate, DNA should be eluted from beads in 10 mM Tris-HCl (pH 8.0 – 8.5). Elution of DNA in PCR-grade water is not recommended, as DNA is unstable in unbuffered solutions.

Input DNA

- This protocol has been validated for library construction from 100 ng – 1 µg of appropriately fragmented, double-stranded DNA, but 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.
- If input DNA is quantified before fragmentation, and/or fragmented DNA is subjected to a bead-based 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 converted to adapter-ligated molecules is typically between 5% and 15%. This applies to high-quality DNA and can be significantly lower for DNA of lower quality, e.g., FFPE samples. Workflows that contain additional bead-based cleanups or size selection prior to library amplification are likely to result in a lower yield of adapter-ligated molecules.
- Solutions containing high concentrations of EDTA and strong buffers may negatively affect the end repair reaction, and should be avoided. Fragmentation of DNA in water is not recommended.

Table 1. Recommended adapter concentrations (10 µL of stock per 70 µL ligation and nick repair reaction)

Insert DNA per reaction	Recommended adapter concentration for DNA with a median fragment size of:							
	130 bp		260 bp		320 bp		410 bp	
	Stock	Final	Stock	Final	Stock	Final	Stock	Final
1 µg	10 µM	1.4 µM	10 µM	1.4 µM	5 µM	0.7 µM	5 µM	0.7 µM
500 ng	5 µM	0.7 µM	5 µM	0.7 µM	2.5 µM	0.36 µM	2.5 µM	0.36 µM
100 ng	1 µM	0.14 µM	1 µM	0.14 µM	0.5 µM	0.07 µM	0.5 µM	0.07 µM

Adapter Concentrations

- The recommended adapter concentration is dependent on the amount of input DNA, and the median fragment size of the library. As a general guideline, an adapter:insert molar ratio between 10:1 and 20:1 is recommended.
- The recommended adapter concentrations for 130, 260, 320, and 410 bp inserts, prepared from 100 ng – 1 µg of input DNA, are provided in Table 1.
- KAPA Adapters are supplied at a concentration of 10 µM. When 10 µL of each adapter is used per 70 µL ligation and nick repair reaction, the final concentration of each adapter is 1.4 µM.
- If a lower final concentration is required, a dilution of the 10 µM adapters to the appropriate concentration is recommended, such that addition of 10 µL of each diluted adapter to a 70 µL ligation and nick repair reaction will result in the recommended final adapter concentration, as shown in Table 1.
- While it is not necessary to adjust adapter concentrations to accommodate moderate sample-to-sample variation in input DNA quantity, the use of an adapter concentration that is appropriate for the molar concentration of input DNA is recommended. It is important to maintain an adapter:insert ratio of ≥10:1 in order to minimize the formation of chimeric library inserts.

Post-ligation Processing

- Ion Torrent adapters do not readily ligate to form dimers as they are not 5' phosphorylated. A single cleanup after ligation and nick repair may therefore be sufficient, as unligated adapters are unlikely to interfere in qPCR-based library quantification and template preparation (emPCR). However, for complete removal of all adapter products after the ligation and nick repair reaction, two bead-based cleanups are required.
- Ligation and Nick Repair Buffer (5X) contains a high concentration of PEG 6000, which will interfere with efficient size selection. If size selection is performed between adapter ligation and library amplification (or template preparation), a single post-ligation cleanup with KAPA Pure Beads is recommended.
- The volume used to resuspend the washed beads after the post-ligation cleanup(s) should be adjusted to suit your chosen workflow:
  - If proceeding directly to library amplification, you should determine an appropriate final volume in which to elute the library DNA. You may wish to divert and/or reserve some of this library material for archival and/or QC purposes. A single 50 µL library amplification reaction as described in this protocol usually yields ~1 µg of DNA, and requires 20 µL of input DNA; a final elution volume of 22 – 25 µL is therefore recommended to allow for some liquid remaining in the tube.
  - If proceeding with size selection, elute the library DNA in an appropriate volume according to the chosen size selection method.

Size Selection

- Size selection requirements vary widely according to specific applications. Size selection may be achieved by means of a variety of common methods including:
  - double-sided, bead-based size selection,
  - manual agarose gel electrophoresis, excision and purification, and
  - automated DNA size selection (e.g., Sage Science Pippin Prep™).
- Size selection inevitably leads to a loss of sample material. Depending on the details, these losses can be dramatic (>80%), significantly increasing the number of amplification cycles needed to generate sufficient material for 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, may eliminate the need for size selection, thereby simplifying the library construction process and limiting sample losses.

- While size selection is usually carried out after adapter ligation and before library amplification, it may be inserted at alternative points in the overall workflow as follows:
  - prior to end repair of fragmented DNA, or
  - after library amplification.
- This protocol provides recommendations for double-sided size selection of 200, 330, 390, and 480 bp libraries. However, size selection is sensitive to multiple factors and any size selection protocol should be carefully optimized and validated before it is used for precious samples.

Library Amplification

- The enzyme provided in KAPA HiFi HotStart ReadyMix (2X) is an antibody-based hot start formulation of KAPA HiFi DNA Polymerase, a novel B-family DNA polymerase engineered for increased processivity and high fidelity. KAPA HiFi HotStart 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.
- Excessive library amplification can result in unwanted artifacts such as PCR duplicates, chimeric library inserts, amplification bias, and heteroduplex formation. It is generally best to limit the extent of library amplification as far as possible, while ensuring that sufficient material is generated for QC and downstream processing (e.g., target enrichment or sequencing). The effects of over-amplification are demonstrated in Figures 1 and 2.
- In general, libraries prepared from 1 µg input gDNA do not require amplification, while libraries prepared from less than 100 ng do require amplification. To determine whether samples require amplification, quantify the libraries with the KAPA Library Quantification Kit for Ion Torrent.
- Table 2 provides typical library yield and concentration following library amplification of adapter-ligated, non-size-selected libraries prepared from 100 ng and 1 µg DNA, for various numbers of PCR cycles.

Note: size selection and other factors related to a particular workflow will determine the actual amount of adapter-ligated library DNA available for PCR amplification.

- If cycled to completion (not recommended), a single 50 µL library amplification PCR, performed as described in this protocol, can produce ~1.5 – 2 µg of amplified library (30 – 40 ng/µL). To minimize over-amplification and associated undesired artifacts, the number of amplification cycles should be optimized to produce ~0.5 – 1.5 µg of amplified library (10 – 30 ng/µL).
- If it is necessary to obtain more DNA, first ensure that the reaction is fully optimized, and then perform multiple 50 µL reactions per sample rather than increasing the PCR volume.
- Figure 1 illustrates typical yields following library amplification for various numbers of PCR cycles as quantified by qPCR, Bioanalyzer and Quant-IT™ PicoGreen®. Yields calculated by qPCR reach a plateau at ~2 µg total yield per 50 µL. Conversely, library concentrations determined using Bioanalyzer and Quant-IT™ PicoGreen® assays show a different pattern and appear to be less reliable for libraries amplified for greater numbers of PCR cycles.
- This discrepancy is likely due to the depletion of primers and/or dNTPs in later PCR cycles. Under these conditions, single-stranded DNA molecules are no longer efficiently converted to double-stranded DNA, and instead dissociate during the thermal denaturation step and then anneal imperfectly to antisense strands that are only partially complementary. This presumably results in the formation of so-called “daisy chains” comprising large assemblies of improperly annealed, partially double-stranded, heteroduplex DNA. The Quant-IT PicoGreen assay is specific for double-stranded DNA and thus the single-stranded component of “daisy chain” molecules in over-amplified samples leads to under-estimation of the DNA concentration.
- Quantification using a double-stranded DNA-specific Bioanalyzer assay (e.g., the Agilent® High Sensitivity DNA Kit; see Figure 2) is also problematic for such samples, as equivalent masses of single- and double-stranded DNA likely do not produce the same amount of fluorescence. Additionally, the heteroduplex library fragments migrate slowly during electrophoresis and are represented as high molecular weight species, leading to difficulties in setting baselines and estimating the true average library size.

Table 2. Predicted library amplification yield

PCR cycles	Recovered yield per 50 µL library amplification reaction for	
	100 ng fragmented input DNA	1 µg fragmented input DNA
3	50 ng/5 nM	500 ng/54 nM
6	400 ng/43 nM	1600 ng/173 nM
8	1500 ng/162 nM	1700 ng/183 nM
10	1600 ng/173 nM	1700 ng/183 nM
15	1900 ng/205 nM	1800 ng/194 nM
20	2100 ng/227 nM	1800 ng/194 nM

Library amplification reactions were cleaned up using one volume of AMPure® XP and eluted in 50 µL. Concentrations were determined by qPCR using the KAPA Library Quantification Kit for Ion Torrent, and molar concentrations were calculated assuming an average fragment length of 300 bp.

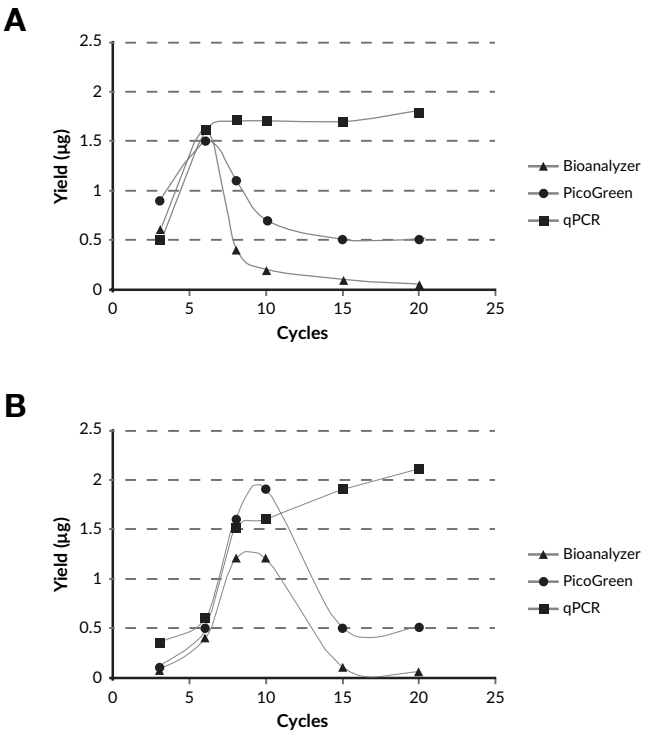

Figure 1. Yield of PCR-amplified libraries as determined by three quantification methods. Library amplification was performed with increasing numbers of PCR cycles according to the recommended protocol, using libraries prepared from 1 µg (**A**) or 100 ng (**B**) of input DNA. Reactions were cleaned up using one volume of AMPure XP and eluted in 50 µL. Amplified library concentrations were determined by Bioanalyzer, Quant-IT™ PicoGreen®, or qPCR (KAPA Library Quantification Kit).

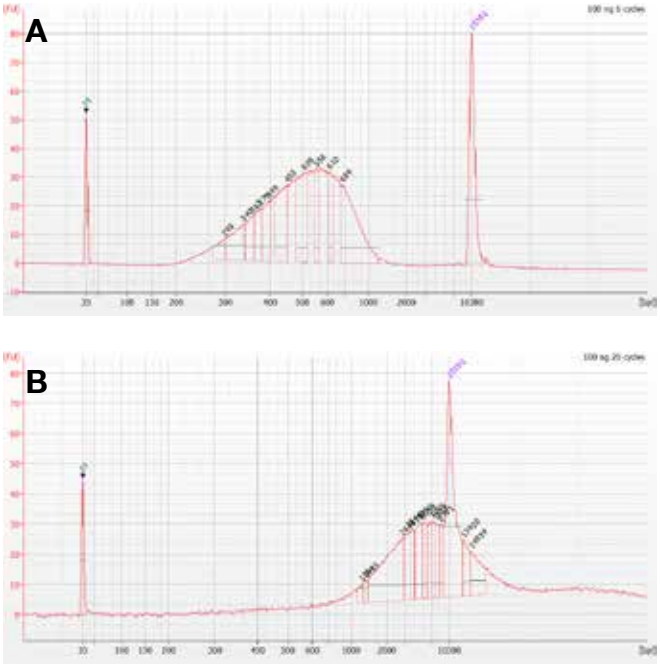

Figure 2. Excessive library amplification leads to high molecular weight DNA heteroduplexes, or "daisy chains". Library amplification reactions of libraries prepared from 100 ng DNA were cleaned up using one volume of AMPure XP after library amplification for 6 cycles (**A**) or 20 cycles (**B**). Libraries were analysed using the Agilent® Bioanalyzer High Sensitivity DNA Kit.

Assessing the Library and Proceeding to Template Preparation

- A specific library construction workflow should be tailored and optimized to yield a sufficient amount of adapter-ligated molecules of the desired size distribution for template preparation and sequencing, as well as for QC and archiving purposes.
- Library size distribution, and the absence of primer dimers and/or over-amplification products, should be confirmed by means of an electrophoretic method.
- To determine the required library dilution for template preparation, library quantification can be performed by using the KAPA Library Quantification Kit for Ion Torrent.

Library Preparation Master Mixes

The recommended master mixes to be prepared for end repair, ligation and nick repair, and amplification are given in Tables 3 to 5. Table 6 provides the required volumes of additional reagents not supplied in this kit.

Table 3. Composition of the end repair reaction

Component:	1 library	8 libraries *Inc. 10% excess*	48 libraries *Inc. 23% excess*
End repair master mix:			
Water	8 µL	70 µL	472 µL
End Repair Buffer (10X)	7 µL	62 µL	413 µL
End Repair Enzyme Mix	5 µL	44 µL	295 µL
Total master mix volume:	**20 µL**	**176 µL**	**1.18 mL**
Final reaction composition:	**Per reaction**		
Fragmented DNA	X µL		
Balance of water required	50-X µL		
Total reaction volume:	**70 µL**		

Table 4. Composition of the ligation and nick repair reaction

Component:	1 library	8 libraries *Inc. 10% excess*	48 libraries *Inc. 23% excess*
Ligation and nick repair master mix:			
Ligation and Nick Repair Buffer (5X)	14 µL	123 µL	826 µL
Ligation and Nick Repair Enzyme	6 µL	53 µL	354 µL
Total master mix volume:	**20 µL**	**176 µL**	**1.18 ml**
Final reaction composition:	**Per reaction**		
End-repaired DNA	30 µL		
Adapter P1	10 µL		
Adapter A	10 µL		
Total reaction volume:	**70 µL**		

Table 5. Composition of the library amplification reaction

Component:	1 library	8 libraries *Inc. 10% excess*	48 libraries *Inc. 23% excess*
Library amplification master mix:			
KAPA HiFi HotStart ReadyMix (10X)	25 µL	220 µL	1.48 mL
Total master mix volume:	**25 µL**	**220 µL**	**1.48 mL**
Final reaction composition:	**Per reaction**		
Adapter-ligated library DNA	20 µL		
Primer premix (10X) (5 µM each primer)	5 µL		
Total reaction volume:	**50 µL**		

Table 6. Approximate volumes of additional reagents required

Component:	1 library	8 libraries *Inc. 10% excess*	48 libraries *Inc. 23% excess*
KAPA Pure Beads (sold separately):			
End repair cleanup	120 µL	~1.1 mL	~7.1 mL
Ligation and nick repair cleanups	180 µL	~1.6 mL	~10.7 mL
Amplification cleanup	70 µL	~0.6 mL	~4.2 mL
Total volume required:	**370 µL**	**~3.3 mL**	**~22 mL**
Component:	**1 library**	**8 libraries** *Inc. 10% excess*	**48 libraries** *Inc. 23% excess*
80% ethanol (not supplied):			
End repair cleanup	400 µL	~3.5 mL	~23.6 mL
Ligation and nick repair cleanups	800 µL	~7.1 mL	~47.2 mL
Amplification cleanup	400 µL	~3.5 mL	~23.6 mL
Total volume required:	**1.6 mL**	**~14.1 mL**	**~94.4 mL**

Library Preparation Protocol

1. End Repair Reaction Setup

1.1 Assemble each end repair reaction as follows:

Component	Volume
Fragmented, double-stranded DNA	50 µL
End repair master mix (Table 3)	20 µL
Total volume:	**70 µL**

1.2 Mix and incubate at 20ºC for 30 min.

1.3 Proceed immediately to **End Repair Cleanup** (step 2).

2. End Repair Cleanup

2.1 To each 70 µL end repair reaction, add:

Component	Volume
KAPA Pure Beads	120 µL
Total volume:	**190 µL**

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

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

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

2.5 Carefully remove and discard the supernatant.

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

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

2.8 Carefully remove and discard the ethanol.

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

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

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

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

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

2.14 Resuspend the beads in 35 µL of elution buffer (10 mM Tris-HCl, pH 8.0 – 8.5) by vortexing thoroughly, or by pipetting up and down multiple times.

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

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

2.17 Transfer 30 µL supernatant to a new plate/tube(s) and proceed with **Adapter Ligation and Nick Repair Reaction Setup** (step 3).

SAFE STOPPING POINT

If you are not proceeding immediately to **Adapter Ligation and Nick Repair Reaction Setup** (step 3), the protocol can be stopped here. Store at 4°C or -20°C overnight.

3. Adapter Ligation and Nick Repair Reaction Setup

3.1 To each plate/tube(s) containing 30 µL of end-repaired DNA, add:

Component	Volume
Ligation and nick repair master mix (Table 4)	20 µL
Adapter P1 (required conc. see Table 1)	10 µL
Adapter A (required conc. see Table 1)	10 µL
Total volume:	**70 µL**

3.2 Mix and incubate the plate/tube(s) at 20°C for 15 min, followed by 65°C for 5 min.

3.3 Proceed immediately with **Ligation and Nick Repair Cleanup** (step 4).

4. Ligation and Nick Repair Cleanup

Depending on requirements and chosen workflow, either one or two post-ligation cleanups should be performed. Consult **Important Parameters: Post-ligation Processing** (p. 4) for more information.

4A. 1st Post-ligation Cleanup

4A.1 To each 70 µL ligation and nick repair reaction, add:

Component	Volume
KAPA Pure Beads	70 µL
Total volume:	**140 µL**

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

4A.3 Incubate the plate/tube(s) at room temperature for 5 – 15 min to bind DNA to the beads.

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

4A.5 Carefully remove and discard the supernatant.

4A.6 Keeping the plate/tube(s) on the magnet, add 200 µL of 80% ethanol.

4A.7 Incubate the plate/tube(s) on the magnet at room temperature for ≥30 sec.

4A.8 Carefully remove and discard the ethanol.

4A.9 Keeping the plate/tube(s) on the magnet, add 200 µL of 80% ethanol.

4A.10 Incubate the plate/tube(s) on the magnet at room temperature for ≥30 sec.

4A.11 Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.

4A.12 Dry the beads at room temperature for 3 – 5 min, or until all of the ethanol has evaporated. ***Caution: over-drying the beads may result in reduced yield.***

4A.13 Remove the plate/tube(s) from the magnet.

4A.14 Thoroughly resuspend the beads in an appropriate volume of 10 mM Tris-HCl (pH 8.0 – 8.5). Incubate the plate/tube(s) at room temperature for 2 min to elute the DNA off the beads. Suggested volumes are as follows:

- If proceeding to a second post-ligation cleanup, resuspend the beads in 52 µL, place plate/tube(s) on the magnet and transfer 50 µL of the supernatant to a new plate/tube(s). Proceed to **2nd Post-ligation Cleanup** (step 4B).
- If proceeding to double-sided size selection, resuspend the beads in 105 µL, place plate/tube(s) on the magnet and transfer 100 µL of the supernatant to a new plate/tube(s). Proceed to **Double-sided Size Selection** (step 5).
- If proceeding to amplification or template preparation, resuspend the beads in 25 µL, place plate/tube(s) on the magnet and transfer the clear supernatant to a new plate/tube(s). Proceed with **Library Amplification** (step 6) or template preparation, as appropriate.

4B. 2nd Post-ligation Cleanup (optional)

4B.1 To each 50 µL of supernatant from the first ligation and nick repair reaction cleanup, add:

Component	Volume
KAPA Pure Beads	70 µL
Total volume:	**120 µL**

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

4B.3 Incubate the plate/tube(s) at room temperature for 5 – 15 min to bind DNA to the beads.

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

4B.5 Carefully remove and discard the supernatant.

4B.6 Keeping the plate/tube(s) on the magnet, add 200 µL of 80% ethanol.

4B.7 Incubate the plate/tube(s) on the magnet at room temperature for ≥30 sec.

4B.8 Carefully remove and discard the ethanol.

4B.9 Keeping the plate/tube(s) on the magnet, add 200 µL of 80% ethanol.

4B.10 Incubate the plate/tube(s) on the magnet at room temperature for ≥30 sec.

4B.11 Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.

4B.12 Dry the beads at room temperature for 3 – 5 min, or until all of the ethanol has evaporated. ***Caution: over-drying the beads may result in reduced yield.***

4B.13 Thoroughly resuspend the beads in 25 µL of 10 mM Tris-HCl (pH 8.0 – 8.5).

4B.14 Incubate the plate/tube(s) at room temperature for 2 min to elute the DNA off the beads.

4B.15 Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.

4B.16 Transfer the clear supernatant to a new plate/tube(s) and proceed with **Library Amplification** (step 6) or template preparation, as appropriate.

5. Double-sided Size Selection (optional)

5.1 To 100 µL of DNA in 10 mM Tris-HCl (pH 8.0 – 8.5), add:

Component	Target library size (including adapters)			
	200 bp	330 bp	390 bp	480 bp
KAPA Pure Beads	90 µL	70 µL	60 µL	45 µL
Total volume:	**190 µL**	**170 µL**	**160 µL**	**145 µL**

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

5.3 Incubate the plate/tube(s) at room temperature for 5 – 15 min to allow larger library fragments to bind to the beads.

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

5.5 Carefully transfer the supernatant containing the smaller library fragments to new plate/tube(s), taking care not to transfer any beads with the supernatant.

5.6 Discard the old plate/tube(s) containing the larger library fragments.

5.7 To the supernatant in the new plate/tube(s) add:

Component	Target library size (including adapters)			
	200 bp	330 bp	390 bp	480 bp
Supernatant	~185 µL	~165 µL	~155 µL	~140 µL
KAPA Pure Beads	20 µL	20 µL	20 µL	20 µL
Total volume:	**~205 µL**	**~185 µL**	**~175 µL**	**~160 µL**

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

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

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

5.11 Carefully remove and discard the supernatant.

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

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

5.14 Carefully remove and discard the ethanol.

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

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

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

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

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

5.20 Thoroughly resuspend the beads in 25 µL of 10 mM Tris-HCl (pH 8.0 – 8.5).

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

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

5.23 Transfer the clear supernatant to a new plate/tube(s) and proceed with **Library Amplification** (step 6) or template preparation, as appropriate.

6. Library Amplification Reaction Setup

Please refer to **Important Parameters** (p. 3) for more information on optimizing library amplification.

6.1 Assemble each library amplification reaction as follows:

Component	Volume
Water (if required)	0 µL
Library DNA	20 µL
KAPA HiFi HotStart ReadyMix (2X)	25 µL
Primer premix (10X) (5 µM each primer)*	5 µL
Total volume:	**50 µL**

*The recommended final concentration of each primer in the library amplification reaction is 500 nM.

6.2 Perform PCR with the following thermocycling parameters:

Step	Temp	Duration	Cycles
Initial denaturation	98ºC	45 sec	1
Denaturation	98ºC	15 sec	As required*
Annealing	65ºC	30 sec	
Extension	72ºC	30 sec	
Final extension	72ºC	60 sec	1
HOLD	4ºC	∞	1

*Refer to Table 2 and Figure 1.

6.3 Proceed directly to **Library Amplification Cleanup** (step 7).

7. Library Amplification Cleanup

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

Component	Volume
KAPA Pure Beads	70 µL
Library amplification reaction product	50 µL
Total volume:	**120 µL**

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

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

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

7.5 Carefully remove and discard the supernatant.

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

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

7.8 Carefully remove and discard the ethanol.

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

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

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

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

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

7.14 Resuspend the beads in an appropriate volume of 10 mM Tris-HCl (pH 8.0 – 8.5) and mix thoroughly by vortexing, or by pipetting up and down multiple times.

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

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

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

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