KAPABIOSYSTEMS

Technical Data Sheet

KAPA Stranded mRNA-Seq Kit

Illumina® Platforms

KR0960 – v7.20

This Technical Data Sheet provides product information and a detailed protocol for the KAPA Stranded mRNA-Seq Kit for Illumina platforms.

The document applies to KAPA Stranded mRNA-Seq Kits (07962193001 and 07962207001) and KAPA mRNA Capture Kits (07962231001 and 07962240001).

Contents

Product Description . . . 2

Product Applications . . . 2

Product Specifications . . . 2

- Shipping and Storage . . . 2
- Handling . . . 2
- Quality Control . . . 2

Safety Information . . . 3

- Precautions . . . 3
- Waste handling . . . 3

Important Parameters . . . 3

- Input RNA Requirements . . . 3
- RNA Handling . . . 3
- RNA Fragmentation . . . 4
- Safe Stopping Points . . . 4
- Reaction Setup . . . 4
- Reaction Cleanups . . . 4
- Adapter Design and Concentration . . . 5
- Library Amplification . . . 5
- Evaluating the Success of Library Construction . . . 6

Process Workflow . . . 7

Library Construction Protocol . . . 8

Restrictions and Liabilities . . . 16

Note to Purchaser: Limited Product Warranty . . . 16

Note to Purchaser: Limited License . . . 16

Kapa/*Roche* Kit Codes and Components		
KK8420 *07962193001* (24 libraries)	mRNA Capture Beads	1.3 mL
	mRNA Bead Binding Buffer	3.9 mL
	mRNA Bead Wash Buffer	9.6 mL
	RNase-free Water	6.5 mL
	Fragment, Prime and Elute Buffer (2X)	264 μL
	1st Strand Synthesis Buffer	264 μL
	KAPA Script	25 μL
	2nd Strand Marking Buffer	750 μL
	2nd Strand Synthesis Enzyme Mix	50 μL
	A-Tailing Buffer (10X)	80 μL
	A-Tailing Enzyme	80 μL
	Ligation Buffer (5X)	380 μL
	DNA Ligase	135 μL
	PEG/NaCl Solution	5 mL
	Library Amplification Primer Mix (10X)	138 μL
	KAPA HiFi HotStart ReadyMix (2X)	690 μL
KK8421 *07962207001* (96 libraries)	mRNA Capture Beads	5.1 mL
	mRNA Bead Binding Buffer	15.3 mL
	mRNA Bead Wash Buffer	40 mL
	RNase-free Water	25 mL
	Fragment, Prime and Elute Buffer (2X)	1.32 mL
	1st Strand Synthesis Buffer	1.32 mL
	KAPA Script	120 μL
	2nd Strand Marking Buffer	3.72 mL
	2nd Strand Synthesis Enzyme Mix	240 μL
	A-Tailing Buffer (10X)	650 μL
	A-Tailing Enzyme	360 μL
	Ligation Buffer (5X)	1.7 mL
	DNA Ligase	600 μL
	PEG/NaCl Solution	30 mL
	Library Amplification Primer Mix (10X)	600 μL
	KAPA HiFi HotStart ReadyMix (2X)	3 mL
KK8440 *07962231001* (24 libraries)	mRNA Capture Beads	1.2 mL
	mRNA Bead Binding Buffer	3.6 mL
	mRNA Bead Wash Buffer	9.6 mL
	RNase-free Water	6.5 mL
KK8441 *07962240001* (96 libraries)	mRNA Capture Beads	4.8 mL
	mRNA Bead Binding Buffer	14.4 mL
	mRNA Bead Wash Buffer	40 mL
	RNase-free Water	25 mL

Quick Notes

- This protocol is suitable for the isolation of intact poly(A) RNA from 100 ng – 4 μg of total RNA.
- Accurate strand origin information is retained in >99% of unique mapped reads.
- To prevent foaming, avoid shaking or high-speed vortexing of mRNA capture beads.
- This kit contains all the reagents needed for library construction, and high efficiency and low bias library amplification except for adapters and beads. KAPA Pure Beads and KAPA Adapters are sold separately.
- PEG/NaCl Solution is provided for "with-bead" reaction cleanups.
- Not compatible with small RNAs <100 bp in length.

Product Description

The KAPA Stranded mRNA-Seq Kit contains all of the buffers and enzymes required for poly(A) mRNA capture and construction of stranded mRNA-seq libraries from 100 ng – 4 µg of intact, total RNA via the following steps:

1. mRNA capture using magnetic oligo-dT beads;
2. fragmentation using heat and magnesium;
3. 1st strand cDNA synthesis using random priming;
4. 2nd strand synthesis and marking, which converts the cDNA:RNA hybrid to double-stranded cDNA (dscDNA), and incorporates dUTP into the 2nd cDNA strand;
5. A-tailing, to add dAMP to the 3'-ends of the dscDNA library fragments;
6. adapter ligation, where dsDNA adapters with 3'-dTMP overhangs are ligated to A-tailed library insert fragments; and
7. library amplification, to amplify library fragments carrying appropriate adapter sequences at both ends using high-fidelity, low-bias PCR. The strand marked with dUTP is not amplified, allowing strand-specific sequencing.

This kit provides all of the enzymes and buffers required for mRNA enrichment, cDNA synthesis, and library construction and amplification, but does not include RNA, adapters, or beads. KAPA Pure Beads and KAPA Adapters are sold separately. Reaction buffers are supplied in convenient formats comprising all of the required reaction components. This minimizes the risk of RNase contamination, ensures consistent and homogenous reaction composition, and improves uniformity among replicate samples. Similarly, a single enzyme mixture is provided for each step of the library construction process, reducing the number of pipetting steps.

In order to maximize sequence coverage uniformity and to maintain relative transcript abundance, it is critical that library amplification bias be kept to a minimum. KAPA HiFi DNA Polymerase is designed for low-bias, high-fidelity PCR, and is the polymerase of choice for NGS library amplification.[1,2,3,4] KAPA Stranded mRNA-Seq Kits include KAPA HiFi HotStart ReadyMix (2X) and Library Amplification Primer Mix (10X) for library amplification.

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 Stranded mRNA-Seq Kit is designed for both manual and automated NGS library construction from 100 ng – 4 µg of intact, total RNA. The protocol is applicable to a wide range of RNA-seq applications, including:

- gene expression
- single nucleotide variation (SNV) discovery
- splice junction and gene fusion identification
- characterization of polyadenylated RNAs.

Product Specifications

Shipping and Storage

KAPA Stranded mRNA-Seq Kits are supplied in two boxes. Box 1 contains capture beads and buffers, and is shipped on dry ice or ice packs, depending on the destination country. ***Upon receipt, store Box 1 at 2°C to 8°C.*** Box 2 contains enzymes and buffers for cDNA synthesis and library preparation, and is shipped on dry ice or ice packs, depending on the destination country. The contents of Box 2 are temperature sensitive, and appropriate care should be taken during storage. ***Upon receipt, store Box 2 at -15°C to -25°C*** in a constant temperature freezer. The 1st Strand Synthesis Buffer and PEG/NaCl Solution supplied in Box 2 are light sensitive, and should be protected from light during storage. 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

Reagents for mRNA capture (Box 1) ***must be stored at 2°C to 8°C, and not at -15°C to -25°C***, as long-term freezing will damage the magnetic capture beads. The mRNA Capture Beads and mRNA Bead Binding Buffer may form a precipitate when refrigerated; this is normal and does not affect product performance. Equilibrate reagents for mRNA capture to room temperature and mix thoroughly before use. mRNA Capture Beads and mRNA Bead Binding Buffer contain detergent. To prevent excessive foaming, high-speed vortexing and vigorous shaking should be avoided.

Reagents for cDNA synthesis and library preparation (Box 2) ***must be stored at -15°C to -25°C***, as these components are temperature sensitive. Ensure that all cDNA synthesis and library preparation reagents have been fully thawed and thoroughly mixed before use. Keep all reaction components and master mixes on ice whenever possible during handling and preparation.

The 1st Strand Synthesis Buffer and PEG/NaCl Solution are light sensitive, and appropriate care must be taken to minimize light exposure. Similar care should be observed for the 1st Strand Synthesis Master Mix.

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.

PEG/NaCl Solution does not freeze at -15°C to -25°C, but should be equilibrated to room temperature and mixed thoroughly before use. For short-term use, the PEG/NaCl Solution may be stored at 2°C to 8°C (protected from light) for ≤2 months.

Quality Control

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

Safety Information

Precautions

- Handle all samples as if potentially infectious, using safe laboratory procedures. As the sensitivity and titer of potential pathogens in the sample material can vary, the operator must optimize pathogen inactivation and follow the appropriate measures according to local safety regulations.
- Do not eat, drink, or smoke in the laboratory area.
- Do not pipette by mouth.
- Wear protective disposable gloves, laboratory coats, and eye protection, when handling samples and kit reagents.
- Wash hands thoroughly after handling samples and reagents.

Waste handling

- Discard unused reagents and waste in accordance with country, federal, state, and local regulations.
- Safety Data Sheets (SDS) are available online on dialog.roche.com, or upon request from the local Roche office.

Important Parameters

Input RNA Requirements

- This protocol has been validated for library construction from 100 ng – 4 µg intact, total RNA, in ≤50 µL of RNase-free water.
- The quantity and quality of mRNA in a total RNA preparation can vary significantly between samples. An input of 100 ng – 4 µg of total RNA is recommended to ensure that sufficient mRNA is available for downstream library preparation.
- To minimize 3'→5' bias, ensure that RNA is intact, and of high quality. The use of fragmented RNA will result in strong bias towards the 3'-end of the mRNA. To determine the quality of RNA, the sample may be analyzed using an Agilent Bioanalyzer RNA kit. RNA with a RIN score less than 7 is not recommended for this protocol.
- RNA in volumes >50 µL should be concentrated to 50 µL prior to use by either ethanol precipitation, bead purification (e.g., KAPA Pure Beads or RNAClean XP, Beckman Coulter), or column-based methods (e.g., RNeasy MinElute Cleanup Kit, QIAGEN). Note that some loss of material is inevitable when using any of the above methods to concentrate RNA.
- When concentrating RNA, an elution volume of 55 µL in RNase-free water is recommended to ensure that 50 µL is available for use in this protocol.

RNA Handling

- RNases are ubiquitous and special care should be taken throughout the procedure to avoid RNase contamination.
- To avoid airborne RNase contamination, keep all reagents and RNA samples closed when not in use.
- Use a laminar flow hood if available, or prepare a sterile and RNase-free area. Clean the workspace, pipettes and other equipment with an RNase removal product (e.g., RNase*Zap*, Ambion Inc.) according to manufacturer's recommendations.
- To avoid RNase contamination, always wear gloves when handling reagents, and use certified RNase-free plastic consumables. Change gloves after making contact with equipment or surfaces outside of the RNase-free working area.
- To mix samples containing RNA, gently pipette the reaction mixture several times. Vortexing may fragment the RNA, resulting in lower quantity and a reduced library insert size.
- To avoid degradation, minimize the number of freeze-thaw cycles and always store RNA in RNase-free water.

mRNA Capture Beads

- Beads and buffers must be stored at 2°C to 8°C.

- Before use, mRNA Capture Beads must be washed and resuspended in mRNA Bead Binding Buffer.
- When preparing multiple libraries, beads may be washed in batches. A single 1.5 mL tube can accommodate beads for up to 24 libraries. If more than 24 libraries must be prepared, wash the beads in multiple batches.
- When washing a large volume of beads, allow sufficient time for all the beads to collect on the magnet before removing the supernatant.
- Beads will settle gradually; ensure that they are fully resuspended before use.
- mRNA Capture Beads and mRNA Bead Binding Buffer contain detergent. ***High-speed vortexing and vigorous shaking should be avoided to prevent excessive foaming.*** Beads may be resuspended by:
  - vortexing at low to medium speed;
  - gentle pipetting, taking care not to aspirate air; or
  - slow mixing on a tube/bottle roller.
- Before adding the Fragment, Prime and Elute Buffer to the beads, ensure that all of the mRNA Bead Wash Buffer has been removed. Carryover of mRNA Bead Wash Buffer may inhibit 1st strand cDNA synthesis.

RNA Fragmentation

- RNA is fragmented by incubating at a high temperature in the presence of magnesium before carrying out 1st strand cDNA synthesis.
- After RNA fragmentation, immediately place the heat-treated sample on the magnet, and remove the supernatant as soon as the liquid has cleared. Failure to do so may result in rebinding of poly-adenylated regions of RNA to the capture beads, resulting in a loss of transcript coverage.
- Fragmentation conditions given in the **Library Construction Protocol** should be used as a guideline. It is recommended that a non-precious, representative sample of RNA be evaluated for the optimal fragmentation conditions.

Safe Stopping Points

The library construction process from mRNA capture through library amplification can be performed in 8 – 10 hrs, depending on the number of samples being processed and experience. If necessary, the protocol may be paused safely at the following steps:

- After **mRNA Capture** (steps 2.7 – 3.2), the resuspended beads (in 22 µL of Fragment, Prime and Elute Buffer) may be stored at 2°C to 8°C for ≤24 hrs.
- After **2nd Strand Synthesis and Marking Cleanup** (steps 6.1 – 6.13), resuspend the washed beads in 15 µL of A-Tailing Buffer (1X), and store at 2°C to 8°C for ≤24 hrs.
- After **1st Post-ligation Cleanup** (steps 9.1 – 9.15), store the resuspended beads at 2°C to 8°C for ≤24 hrs.
- After **2nd Post-ligation Cleanup** (steps 10.1 – 10.17), store the eluted, unamplified library at 2°C to 8°C for ≤1 week, or at -15°C to -25°C for ≤1 month.

DNA and RNA solutions containing beads must not be frozen or stored dry, as this is likely to damage the beads and result in sample loss. To resume the library construction process, centrifuge briefly to recover any condensate, and add the remaining components required for the next enzymatic reaction in the protocol.

To avoid degradation, minimize the number of freeze-thaw cycles, and always store RNA in RNase-free water, and DNA in a buffered solution (10 mM Tris-HCl, pH 8.0 – 8.5).

Reaction Setup

This kit is intended for manual and automated NGS library construction. To enable a streamlined "with-bead" strategy, reaction components should be combined into master mixes, rather than dispensed separately into individual reactions. When processing multiple samples, prepare a minimum of 10% excess of each master mix to allow for small inaccuracies during dispensing. Recommended volumes for 8, 24, and 96 reactions (with excess) are provided in Tables 2 – 6.

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 RNase- and DNase-free. Low RNA- and DNA-binding plastics are recommended. When selecting the most appropriate plastic consumables for your workflow, consider compatibility with:

- the magnet used during bead manipulations;
- vortex mixers and centrifuges, where appropriate; and
- Peltier devices or thermocyclers used for reaction incubations and/or library amplification.

Reaction Cleanups

- This protocol has been validated for use with both KAPA Pure Beads or Agencourt AMPure XP (Beckman Coulter). Solutions and conditions for DNA binding may differ if other beads are used.
- Cleanup steps should be performed in a timely manner to ensure that enzymatic reactions do not proceed beyond optimal incubation times.
- Observe all the storage and handling recommendations for KAPA Pure Beads or Agencourt AMPure XP. Equilibration to room temperature is essential to achieve specified size distribution and yield of libraries.
- Beads will settle gradually; ensure that they are fully resuspended before use.

- ***To ensure optimal DNA recovery, it is critical that the DNA and KAPA Pure Beads are thoroughly mixed*** (by vortexing or extensive up-and-down pipetting) before the DNA binding incubation.
- Bead incubation times are guidelines only, and may be modified/optimized according to current protocols, previous experience, specific equipment, and samples in order to maximize library construction efficiency and throughput.
- The time required for complete capture of beads varies according to the reaction vessel and magnet used. It is important not to discard or transfer any beads with the removal 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 the wash steps. ***Always use freshly prepared 80% ethanol.***
- It is important to remove all ethanol before proceeding with subsequent reactions. However, over-drying of beads may make them difficult to resuspend, and result in a dramatic loss of DNA. With optimized aspiration of ethanol, drying of beads for 3 – 5 min at room temperature should be sufficient. ***Drying of beads at 37°C is not recommended***.
- Where appropriate, DNA should be eluted from beads in elution buffer (10 mM Tris-HCl, pH 8.0 – 8.5). Elution of DNA in PCR-grade water is not recommended, as DNA is unstable in unbuffered solutions. Purified DNA in elution buffer should be stable at 2°C to 8°C for 1 – 2 weeks, or for at least 1 month at -15°C to -25°C. The long-term stability of library DNA at -15°C to -25°C depends on a number of factors, including library concentration. Always use low DNA-binding tubes for long-term storage, and avoid excessive freezing and thawing.

Adapter Design and Concentration

- KAPA Adapters are recommended for use with the KAPA Stranded mRNA-Seq Kit. However, the kit is also compatible with other full-length adapter designs wherein both the sequencing and cluster generation sequences are added during the ligation step, such as those routinely used in TruSeq (Illumina), SeqCap EZ (Roche) and SureSelect XT2 (Agilent) kits, and other similar library construction workflows. Custom adapters that are of similar design and are compatible with "TA-ligation" of dsDNA may also be used, remembering that custom adapter designs may impact library construction efficiency. For assistance with adapter compatibility and ordering, please visit **kapabiosystems.com/support**.
- 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 the above factors and cost.
- Adapter quality has an impact on the effective concentration of adapter available for ligation. Always source the highest quality adapters from a reliable supplier, dilute and store adapters in a buffered solution with the requisite ionic strength, and avoid excessive freezing and thawing of adapter stock solutions.
- To accommodate different adapter concentrations within a batch of samples processed together, it is best to vary the concentrations of adapter stock solutions, and dispense a fixed volume (5 μL) of each adapter. The alternative (using a single stock solution, and dispensing variable volumes of adapter into ligation reactions) is not recommended.
- Adapter-dimer formation may occur when using input amounts lower than the validated range (100 ng). If adapter-dimers are present, as evidenced by a sharp 120 to 140 bp peak in the final library, perform a second 1X bead cleanup post-amplification to remove small products. Adapter-dimer formation can be prevented in future library preparations by reducing the amount of adapter in the ligation reaction.

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 2.8×10^{-7} errors/base, equivalent to 1 error per 3.5×10^{6} nucleotides incorporated.
- The Library Amplification Primer Mix (10X) is designed to eliminate or delay primer depletion during library amplification reactions performed with KAPA HiFi HotStart ReadyMix. The primer mix is suitable for the amplification of all Illumina libraries flanked by the P5 and P7 flow cell sequences. Primers are supplied at a 10X concentration of 20 μM each, and have been formulated as described below. User-supplied primer mixes may be used in combination with incomplete or custom adapters. Please contact Technical Support at sequencing.roche.com/support for guidelines on the formulation of user-supplied library amplification primers.
- To achieve the highest amplification efficiency and avoid primer depletion, it is critical to use an optimal concentration of high-quality primers. Primers should be used at a final concentration of 0.5 – 4 μM each.

- Library amplification primers should be HPLC-purified and modified to include a phosphorothioate bond at the 3'-terminal of each primer (to prevent degradation by the strong proofreading activity of KAPA HiFi HotStart). Always store and dilute primers in a buffered solution (e.g., 10 mM Tris-HCl, pH 8.0 – 8.5), and limit the number of freeze-thaw cycles. To achieve the latter, store primers at 2°C to 8°C for short-term use, or as single-use aliquots at -15°C to -25°C.
- In library amplification reactions (set up according to the recommended protocol), primers are typically depleted before dNTPs. When DNA synthesis can no longer take place due to substrate depletion, subsequent rounds of DNA denaturation and annealing result in the separation of complementary DNA strands, followed by imperfect annealing to non-complementary partners. This presumably results in the formation of so-called “daisy chains” or “tangled knots”, comprising large assemblies of improperly annealed, partially double-stranded, heteroduplex DNA. These species migrate slower and are observed as secondary, higher molecular weight peaks during the electrophoretic analysis of amplified libraries. However, they typically comprise library molecules of the desired length, which are individualized during denaturation prior to cluster amplification or probe hybridization. Since these heteroduplexes contain significant portions of single-stranded DNA, over-amplification leads to the under-quantification of library molecules with assays employing dsDNA-binding dyes. qPCR-based library 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.
- 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.
- If cycled to completion (***not recommended***), one 50 µL library amplification PCR—performed as described in **Library Amplification** (step 11)—can produce 8 – 10 µg of amplified library. To minimize over-amplification and its associated, undesired artifacts, the number of amplification cycles should be tailored to produce the optimal amount of final library required for downstream processes. This is typically in the range of 250 ng – 1.5 µg.
- The number of cycles recommended in Table 1 should be used as a guide for library amplification. Cycle numbers may require adjustment depending on library amplification efficiency and the presence of adapter-dimer.

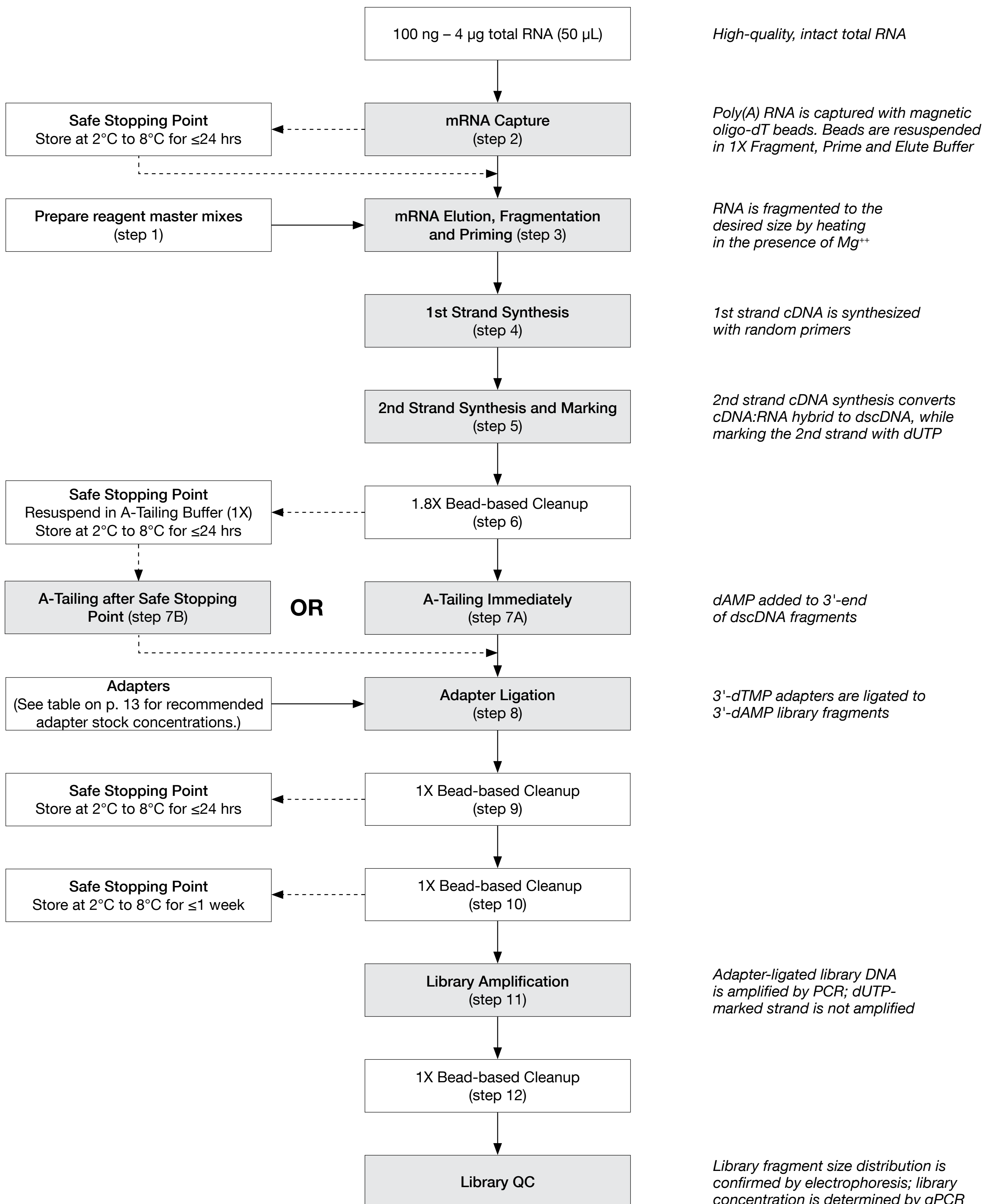
Table 1. Recommended library amplification cycles

Quantity of starting material	Number of cycles
100 – 250 ng	10 – 16
251 – 500 ng	10 – 14
501 – 2000 ng	8 – 12
2001 – 4000 ng	6 – 10

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 sequencing, QC, and archiving purposes.
- The size distribution of pre-capture or final libraries should be confirmed with an electrophoretic method. A LabChip GX, GXII, or GX Touch (PerkinElmer), Bioanalyzer or TapeStation (Agilent Technologies), Fragment Analyzer (Advanced Analytical), or similar instrument is recommended over conventional gels.
- KAPA Library Quantification Kits for Illumina platforms are recommended for qPCR-based quantification of libraries generated using the KAPA HyperPlus workflow. These kits employ primers based on the Illumina flow cell oligos, and can be used to quantify libraries that:
    - are ready for flow-cell amplification, and/or
    - were constructed with full-length adapters, once ligation has been completed (i.e., after the post-ligation cleanup or after library amplification cleanup).
- 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.

Process Workflow



Library Construction Protocol

1. Reagent Preparation

This protocol takes 8 – 10 hrs to complete. Ideally, master mixes for the various steps in the process should be prepared as required.

For maximum stability and shelf-life, enzymes and reaction buffers are supplied separately in the KAPA Stranded mRNA-Seq Kit. For a streamlined "with-bead" protocol, a reagent master mix with a minimum of 10% excess is prepared for each of these enzymatic steps, as outlined in Tables 2 – 6.

Volumes of additional reagents required for the KAPA Stranded mRNA-Seq Kit protocol are listed in Table 7.

In some cases, 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.

At the safe stopping point at A-tailing, a portion of the water and reaction buffer are added to the beads for storage at 2°C to 8°C for ≤24 hrs. 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 the master mix after the safe stopping point are provided in Table 4B.

Always ensure that KAPA Pure Beads and PEG/NaCl Solution are fully equilibrated to room temperature before use.

Table 2. 1st strand synthesis

Component	1 library *Inc. 20% excess*	8 libraries *Inc. 20% excess*	24 libraries *Inc. 20% excess*	96 libraries *Inc. 20% excess*
1st strand synthesis master mix:				
1st Strand Synthesis Buffer	11 µL	88 µL	264 µL	1056 µL
KAPA Script	1 µL	8 µL	24 µL	96 µL
Total master mix volume:	**12 µL**	**96 µL**	**288 µL**	**1152 µL**
Final reaction composition:	**Per reaction**			
1st strand synthesis master mix	10 µL			
Fragmented, primed RNA	20 µL			
Total reaction volume:	**30 µL**			

Table 3. 2nd strand synthesis and marking

Component	1 library *Inc. 10% excess*	8 libraries *Inc. 10% excess*	24 libraries *Inc. 10% excess*	96 libraries *Inc. 10% excess*
2nd strand synthesis and marking master mix:				
2nd Strand Marking Buffer	31 µL	248 µL	744 µL	2976 µL
2nd strand synthesis enzyme mix	2 µL	16 µL	48 µL	192 µL
Total master mix volume:	**33 µL**	**264 µL**	**792 µL**	**3168 µL**
Final reaction composition:	**Per reaction**			
2nd strand synthesis and marking master mix	30 µL			
1st strand cDNA	30 µL			
Total reaction volume:	**60 µL**			

Table 4A. A-tailing (uninterrupted protocol)

Component	1 library	8 libraries *Inc. 10% excess*	24 libraries *Inc. 10% excess*	96 libraries *Inc. 10% excess*
A-tailing master mix:				
Water	24 µL	211.2 µL	634 µL	2534 µL
A-Tailing Buffer (10X)	3 µL	26.4 µL	79 µL	317 µL
A-Tailing Enzyme	3 µL	26.4 µL	79 µL	317 µL
Total master mix volume:	**30 µL**	**264.0 µL**	**792 µL**	**3168 µL**
Resuspend beads in a volume of:	**30 µL**			

Table 4B. A-tailing (safe stopping point)

Component	1 library	8 libraries *Inc. 10% excess*	24 libraries *Inc. 10% excess*	96 libraries *Inc. 10% excess*
A-Tailing Buffer (1X) at safe stopping point:				
Water	13.5 µL	118.8 µL	356 µL	1426 µL
A-Tailing Buffer (10X)	1.5 µL	13.2 µL	40 µL	158 µL
Total master mix volume:	**15 µL**	**132.0 µL**	**396 µL**	**1584 µL**
Resuspend beads in a volume of:	**15 µL**			
Component	**1 library**	**8 libraries** *Inc. 10% excess*	**24 libraries** *Inc. 10% excess*	**96 libraries** *Inc. 10% excess*
A-tailing master mix after safe stopping point:				
Water	10.5 µL	92.4 µL	277 µL	1109 µL
A-Tailing Buffer (10X)	1.5 µL	13.2 µL	40 µL	158 µL
A-Tailing Enzyme	3.0 µL	26.4 µL	79 µL	317 µL
Total master mix volume:	**15 µL**	**132.0 µL**	**396 µL**	**1584 µL**
Final reaction composition:	Per reaction			
Beads with dscDNA in A-Tailing Buffer (1X)	15 µL			
A-tailing master mix	15 µL			
Total reaction volume:	**30 µL**			

Table 5. Adapter ligation

Component	1 library	8 libraries *Inc. 10% excess*	24 libraries *Inc. 10% excess*	96 libraries *Inc. 10% excess*
Ligation master mix:				
Water	16 µL	140.8 µL	422 µL	1690 µL
Ligation Buffer (5X)	14 µL	123.2 µL	370 µL	1478 µL
DNA Ligase	5 µL	44.0 µL	132 µL	528 µL
Total master mix volume:	**35 µL**	**308.0 µL**	**924 µL**	**3696 µL**
Final reaction composition:	Per reaction			
Beads with A-tailed DNA	30 µL			
Ligation master mix	35 µL			
Adapter (350 nM – 1400 nM, as appropriate)	5 µL			
Total reaction volume:	**70 µL**			

Table 6. Library amplification

Component	1 library	8 libraries *Inc. 10% excess*	24 libraries *Inc. 10% excess*	96 libraries *Inc. 10% excess*
Library amplification master mix:				
KAPA HiFi HotStart ReadyMix (2X)	25 µL	220 µL	660 µL	2640 µL
Library Amplification Primer Mix (10X)	5 µL	44 µL	132 µL	528 µL
Total master mix volume:	**30 µL**	**264 µL**	**792 µL**	**3168 µL**
Final reaction composition:	Per reaction			
Adapter-ligated library DNA	20 µL			
Library amplification master mix	30 µL			
Balance of water (if required)	0 µL			
Total reaction volume:	**50 µL**			

Table 7. Volumes of additional reagents required

Component	1 library	8 libraries *Inc. 10% excess*	24 libraries *Inc. 10% excess*	96 libraries *Inc. 10% excess*
PEG/NaCl Solution (provided in kit):				
1st post-ligation cleanup	70 µL	620 µL	1.9 mL	7.5 mL
2nd post-ligation cleanup	50 µL	440 µL	1.3 mL	5.9 mL
Total volume required:	**120 µL**	**1060 µL**	**3.2 mL**	**13.4 mL**
Component	**1 library**	**8 libraries** *Inc. 10% excess*	**24 libraries** *Inc. 10% excess*	**96 libraries** *Inc. 10% excess*
KAPA Pure Beads (sold separately):				
2nd strand synthesis and marking cleanup	108 µL	950 µL	2.9 mL	10.5 mL
Library amplification cleanup	50 µL	440 µL	1.3 mL	5.3 mL
Total volume required:	**158 µL**	**1390 µL**	**4.2 mL**	**15.8 mL**
Component	**1 library**	**8 libraries** *Inc. 10% excess*	**24 libraries** *Inc. 10% excess*	**96 libraries** *Inc. 10% excess*
80% ethanol (freshly prepared; not supplied):				
2nd strand synthesis and marking cleanup	0.4 mL	3.5 mL	10.6 mL	42.2 mL
1st post-ligation cleanup	0.4 mL	3.5 mL	10.6 mL	42.2 mL
2nd post-ligation cleanup	0.4 mL	3.5 mL	10.6 mL	42.2 mL
Library amplification cleanup	0.4 mL	3.5 mL	10.6 mL	42.2 mL
Total volume required:	**1.6 mL**	**14 mL**	**42.4 mL**	**168.8 mL**
Component	**1 library**	**8 libraries** *Inc. 10% excess*	**24 libraries** *Inc. 10% excess*	**96 libraries** *Inc. 10% excess*
Elution buffer (10 mM Tris-HCl, pH 8.0 – 8.5; not supplied):				
1st post-ligation cleanup	50 µL	440 µL	1320 µL	5.3 mL
2nd post-ligation cleanup	22 µL	200 µL	590 µL	2.4 mL
Library amplification cleanup	22 µL	200 µL	590 µL	2.4 mL
Total volume required:	**94 µL**	**840 µL**	**2500 µL**	**10.1 mL**

2. mRNA Capture

This protocol requires 100 ng – 4 µg of intact, total RNA in 50 µL of RNase-free water. Degraded or fragmented total RNA will result in significant 3'-bias.

This protocol has been optimized to isolate mature mRNA from total RNA through two subsequent capture steps using the mRNA Capture Beads. Other RNA molecules with homopolymeric adenosine regions may also be isolated.

RNA samples should only be kept on ice where specified in this protocol, since low temperatures may promote non-specific capture, resulting in increased rRNA in the captured mRNA.

Before starting, equilibrate mRNA Capture Beads, mRNA Bead Binding Buffer, mRNA Bead Wash Buffer and Fragment, Prime and Elute Buffer to room temperature.

Before use, beads must be washed with mRNA Bead Binding Buffer (steps 2.1 – 2.5).

2.1 Resuspend the mRNA Capture Beads thoroughly by pipetting up and down gently, or by using a vortex mixer on a low to medium speed setting to prevent foaming. High-speed vortexing or shaking should be avoided to prevent foaming. Refer to **Important Parameters: mRNA Capture Beads** (p. 3) for more information.

2.2 For each library to be prepared, transfer 52.5 µL (50 µL + 5% excess) of the resuspended mRNA Capture Beads into an appropriate tube. When preparing multiple libraries, beads for up to 24 libraries (1,260 µL) may be washed in a single tube. When preparing more than 24 libraries, wash beads in multiple batches. Please refer to **Important Parameters: mRNA Capture Beads** (p. 3) for additional recommendations regarding bulk bead washing.

2.3 Place the tube on a magnet and incubate at room temperature until the solution is clear. Carefully remove and discard the supernatant, and replace it with an equal volume of mRNA Bead Binding Buffer (52.5 µL per library).

2.4 Remove the tube from the magnet and resuspend the beads by pipetting up and down, or by low to medium speed vortexing. Be careful to avoid producing excessive foam.

2.5 Place the tube on the magnet and incubate at room temperature until the solution is clear. Carefully remove and discard the supernatant, and replace it with an equal volume of mRNA Bead Binding Buffer (52.5 µL per library).

2.6 Remove the tube from the magnet and resuspend the beads by pipetting up and down, or by low to medium speed vortexing. Be careful to avoid producing excessive foam.

2.7 For each RNA sample to be captured, transfer 50 µL of resuspended mRNA Capture Beads into individual tubes or wells of a plate.

2.8 To each well/tube, add 50 µL of the appropriate RNA sample (in RNase-free water).

2.9 Mix thoroughly by gently pipetting up and down several times.

2.10 Place the plate/tube(s) in a thermocycler and perform the 1st mRNA capture as follows:

Step	Temp.	Duration
1st mRNA capture	65°C	2 min
Cool	20°C	5 min

2.11 Place the plate/tube(s) containing the mixture of mRNA Capture Beads and RNA on a magnet and incubate at room temperature until the solution is clear. Remove and discard the supernatant.

2.12 Remove the plate/tube(s) from the magnet and resuspend thoroughly in 200 µL of mRNA Bead Wash Buffer by pipetting up and down several times.

2.13 Place the plate/tube(s) on the magnet and incubate at room temperature until the solution is clear. Remove and discard the supernatant.

2.14 Resuspend the beads in 50 µL of RNase-free water.

2.15 Place the plate/tube(s) in a thermocycler and perform the 2nd mRNA capture as follows:

Step	Temp.	Duration
2nd mRNA capture	70°C	2 min
Cool	20°C	5 min

2.16 Add 50 µL of Bead Binding Buffer to the mixture of mRNA Capture Beads and RNA and mix thoroughly by gently pipetting up and down several times.

2.17 Incubate the plate/tube(s) at 20°C for 5 min.

2.18 Place the plate/tube(s) on the magnet and incubate at room temperature until the solution is clear. Remove and discard the supernatant.

2.19 Remove the beads from the magnet and resuspend in 200 µL of mRNA Bead Wash Buffer by pipetting up and down several times.

2.20 Place the plate/tube(s) on the magnet and incubate at room temperature until the solution is clear. Remove and discard the entire volume of supernatant. ***Caution: carryover of mRNA Bead Wash Buffer may inhibit 1st Strand CDNA Synthesis***

3. mRNA Elution, Fragmentation and Priming

3.1 Prepare the required volume of Fragment, Prime and Elute Buffer (1X) at room temperature as follows:

Component	Volume per sample
RNase-free water	11 µL
Fragment, Prime and Elute Buffer (2X)	11 µL
Total volume:	**22 µL**

3.2 Thoroughly resuspend the mRNA Capture Beads with captured mRNA prepared in step 2.20 above in 22 µL of Fragment, Prime and Elute Buffer (1X).

SAFE STOPPING POINT

Resuspended beads with captured mRNA may be stored at 2°C to 8°C for ≤24 hrs. Do not freeze the samples as this will damage the beads. When ready, proceed to step 3.3 below.

3.3 Place the plate/tube(s) in a thermocycler and carry out the fragmentation and priming program given in the table below:

Desired mean library insert size (bp)	Fragmentation
100 – 200	8 min at 94°C
201 – 300	6 min at 94°C
301 – 400	6 min at 85°C

3.4 Immediately place the plate/tube(s) on a magnet to capture the beads, and incubate until the liquid is clear. ***Caution: to prevent hybridization of poly(A)-rich RNA to the capture beads, do not allow the sample to cool before placing on the magnet.***

3.5 Carefully remove 20 µL of the supernatant containing the eluted, fragmented, and primed RNA into a separate plate or tube.

3.6 Place the plate/tube(s) on ice and proceed immediately to **1st Strand Synthesis** (step 4).

4. 1st Strand Synthesis

4.1 On ice, assemble the first strand synthesis reaction as follows:

Component	Volume
Fragmented, primed RNA eluted from beads	20 µL
1st strand synthesis master mix (Table 2)	10 µL
Total volume:	**30 µL**

4.2 Keeping the plate/tube(s) on ice, mix thoroughly by gently pipetting the reaction up and down several times.

4.3 Incubate the plate/tube(s) using the following protocol:

Step	Temp.	Duration
Primer extension	25°C	10 min
1st strand synthesis	42°C	15 min
Enzyme inactivation	70°C	15 min
HOLD	4°C	∞

4.4 Place the plate/tube(s) on ice and proceed immediately to **2nd Strand Synthesis and Marking** (step 5).

5. 2nd Strand Synthesis and Marking

5.1 On ice, assemble the second strand synthesis and marking reaction as follows:

Component	Volume
1st strand cDNA	30 µL
2nd strand synthesis and marking master mix (Table 3)	30 µL
Total volume:	**60 µL**

5.2 Keeping the plate/tube(s) on ice, mix thoroughly by gently pipetting the reaction up and down several times.

5.3 Incubate the plate/tube(s) using the following protocol:

Step	Temp.	Duration
2nd strand synthesis and marking	16°C	60 min
HOLD	4°C	∞

5.4 Proceed immediately to **2nd Strand Synthesis and Marking Cleanup** (step 6).

6. 2nd Strand Synthesis and Marking Cleanup

6.1 Perform a 1.8X bead-based cleanup by combining the following:

Component	Volume
2nd strand synthesis reaction product	60 µL
KAPA Pure Beads	108 µL
Total volume:	**168 µL**

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

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

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

6.5 Carefully remove and discard 160 µL of supernatant.

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

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

6.8 Carefully remove and discard the ethanol.

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

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

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

6.12 Dry the beads at room temperature until all of the ethanol has evaporated. ***Caution: over-drying the beads may result in reduced yield.***

6.13 Proceed immediately to **A-tailing Immediately** (step 7A), or follow the safe stopping point instructions.

SAFE STOPPING POINT

Resuspend the beads in 15 µL A-Tailing Buffer (1X) (Table 4B), cover the reaction and store at 2°C to 8°C for ≤24 hrs. Do not freeze the samples as this will damage the KAPA Pure Beads. When ready, proceed to **A-tailing after Safe Stopping Point** (step 7B).

7. A-tailing

A-tailing is performed either directly after the **2nd Strand Synthesis and Marking Cleanup**, or after the Safe Stopping Point, where beads were resuspended in A-Tailing Buffer (1X) and stored at 2°C to 8°C for ≤24 hrs. Depending on your chosen workflow, proceed with either **A-tailing Immediately** (step 7A) or **A-tailing after Safe Stopping Point** (step 7B).

7A. A-tailing Immediately

7A.1 Assemble the A-tailing reaction as follows:

Component	Volume
Beads with dscDNA	–
A-tailing master mix (Table 4A)	30 µL
Total volume:	**30 µL**

7A.2 Mix thoroughly by pipetting up and down several times.

7A.3 Incubate the plate/tube(s) using the following protocol:

Step	Temp.	Duration
A-tailing	30°C	30 min
Enzyme inactivation	60°C	30 min
HOLD	4°C	∞

7A.4 Proceed immediately to **Adapter Ligation** (step 8).

7B. A-tailing after Safe Stopping Point

7B.1 To resume library preparation, combine the following reagents to perform A-tailing:

Component	Volume
Beads with dscDNA (in A-Tailing Buffer (1X), Table 4B)	15 µL
A-tailing master mix after safe stopping point (Table 4B)	15 µL
Total volume:	**30 µL**

7B.2 Mix thoroughly by pipetting up and down several times.

7B.3 Incubate the plate/tube(s) using the following protocol:

Step	Temp.	Duration
A-tailing	30°C	30 min
Enzyme inactivation	60°C	30 min
HOLD	4°C	∞

7B.4 Proceed immediately to **Adapter Ligation** (step 8).

8. Adapter Ligation

8.1 Dilute adapters in preparation for ligation targeting the following concentrations:

Quantity of starting material	Adapter stock concentration	Final adapter concentration
100 – 250 ng	140 nM	10 nM
251 – 500 ng	350 nM	25 nM
501 – 2000 ng	700 nM	50 nM
2001 – 4000 ng	1400 nM	100 nM

8.2 Set up the adapter ligation reactions as follows:

Component	Volume
Beads with A-tailed DNA	30 µL
Adapter ligation master mix (Table 5)	35 µL
Diluted adapter stock	5 µL
Total volume:	**70 µL**

8.3 Mix thoroughly by pipetting up and down several times to resuspend the beads.

8.4 Incubate the plate/tube(s) at 20°C for 15 min.

8.5 Proceed immediately to **1st Post-Ligation Cleanup** (step 9).

9. 1st Post-ligation Cleanup

9.1 Perform a 1X bead-based cleanup by combining the following:

Component	Volume
Beads with adapter-ligated DNA	70 µL
PEG/NaCl Solution	70 µL
Total volume:	**140 µL**

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

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

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

9.5 Carefully remove and discard 135 µL of supernatant.

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

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

9.8 Carefully remove and discard the ethanol.

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

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

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

9.12 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.***

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

9.14 Thoroughly resuspend the beads in 50 µL of 10 mM Tris-HCl (pH 8.0 – 8.5).

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

SAFE STOPPING POINT

The solution with resuspended beads can be stored at 2°C to 8°C for ≤24 hrs. Do not freeze the beads, as this can result in dramatic loss of DNA. When ready, proceed to **2nd Post-ligation Cleanup** (step 10).

10. 2nd Post-ligation Cleanup

10.1 Perform a 1X bead-based cleanup by combining the following:

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

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

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

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

10.5 Carefully remove and discard 95 µL of supernatant.

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

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

10.8 Carefully remove and discard the ethanol.

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

10.10 Incubate the plate/tube(s) on the magnet 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 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.***

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

10.14 Thoroughly resuspend the beads in 22 µL of 10 mM Tris-HCl (pH 8.0 – 8.5).

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

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

10.17 Transfer 20 µL of the clear supernatant to a new plate/tube and proceed to **Library Amplification** (step 11).

SAFE STOPPING POINT

The purified, adapter-ligated library DNA may be stored at 2°C to 8°C for ≤1 week, or frozen at -15°C to -25°C for ≤1 month. When ready, proceed to **Library Amplification** (step 11).

11. Library Amplification

11.1 Assemble each library amplification reaction as follows:

Component	Volume
Purified, adapter-ligated DNA	20 µL
Library amplification master mix (Table 6)	30 µL
Total volume:	**50 µL**

11.2 Mix well by pipetting up and down several times.

11.3 Amplify the library using the following thermo-cycling profile:

Step	Temp.	Duration	Cycles
Initial denaturation	98°C	45 sec	1
Denaturation	98°C	15 sec	Refer to Table 1
Annealing*	60°C	30 sec	
Extension	72°C	30 sec	
Final extension	72°C	5 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.

11.4 Immediately proceed to **Library Amplification Cleanup** (step 12).

12. Library Amplification Cleanup

12.1 Perform a 1X bead-based cleanup by combining the following:

Component	Volume
Amplified library DNA	50 μL
KAPA Pure Beads	50 μL
Total volume:	**100 μL**

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

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

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

12.5 Carefully remove and discard 95 μL of supernatant.

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

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

12.8 Carefully remove and discard the ethanol.

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

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

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

12.12 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.*

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

12.14 Thoroughly resuspend the dried beads in 22 μL of 10 mM Tris-HCl (pH 8.0 – 8.5).

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

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

12.17 Transfer 20 μL of the clear supernatant to a new plate/tube(s) and store the purified, amplified libraries at 2°C to 8°C for ≤ 1 week or at -15°C to -25°C.

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