KAPABIOSYSTEMS

Technical Data Sheet

KAPA Stranded RNA-Seq Kit with RiboErase (HMR)

Illumina® Platforms

KR1151 – v5.17

This Technical Data Sheet provides product information and a detailed protocol for the KAPA Stranded RNA-Seq Kit with RiboErase (HMR, or Human/Mouse/Rat) for Illumina platforms.

Contents

Kapa/*Roche* Kit Codes and Components		
KK8483 *07962282001* 24 libraries	Hybridization Buffer	110 µL
	Hybridization Oligos (HMR)	110 µL
	Depletion Buffer	80 µL
	RNase H	55 µL
	DNase Buffer	60 µL
	DNase	55 µL
	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
KK8484 *07962304001* 96 libraries	Hybridization Buffer	480 µL
	Hybridization Oligos (HMR)	480 µL
	Depletion Buffer	360 µL
	RNase H	240 µL
	DNase Buffer	264 µL
	DNase	240 µL
	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

Quick Notes

- This protocol is suitable for the depletion of ribosomal RNA from 100 ng – 1 µg of total human, mouse, or rat RNA (HMR).
- Suitable for high- and low-quality RNA samples, including FFPE. Results may vary depending on the input amount and quality.
- 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 RNA-Seq Kit with RiboErase (HMR) contains all of the buffers and enzymes required for depletion of ribosomal RNA (rRNA) followed by construction of stranded RNA-seq libraries from 100 ng – 1 µg of total RNA via the following steps:

1. depletion of rRNA by hybridization of complementary DNA oligonucleotides, followed by treatment with RNase H and DNase to remove rRNA duplexed to DNA and original DNA oligonucleotides, respectively;
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.

The kit provides all of the enzymes and buffers required for rRNA depletion, 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] The KAPA Stranded RNA-Seq Kit with RiboErase (HMR) includes 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 – 11 (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 RNA-Seq Kit with RiboErase (HMR) is designed for both manual and automated NGS library construction from 100 ng – 1 µg of total RNA. The kit depletes both cytoplasmic (5S, 5.8S, 18S, and 28S), and mitochondrial (12S and 16S) rRNA species. The protocol is applicable to a wide range of RNA-seq applications, including:

- gene expression analysis of high- and low-quality RNA samples (e.g., extracted from FFPE tissue)
- single nucleotide variation (SNV) discovery
- splice junction and gene fusion identification
- characterization of non-polyadenylated RNAs, including non-coding and immature RNAs.

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 Stranded RNA-Seq Kits with RiboErase (HMR) are shipped on dry ice. Upon receipt, immediately store enzymes and reaction buffer components at -15°C to -25°C in a constant temperature freezer. The 1st Strand Synthesis Buffer and PEG/NaCl Solution 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

Always ensure that components have been fully thawed and thoroughly mixed before use. Keep all reaction components and master mixes on ice whenever possible during handling and preparation, unless specified otherwise.

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. Reagent kits are functionally validated through construction of transcriptome libraries and sequencing on an NGS platform. Please contact Technical Support at **sequencing.roche.com/support** for more information.

Important Parameters

Input RNA Requirements

- This protocol has been validated for library construction from 100 ng – 1 µg total RNA, in 10 µL of RNase-free water.
- The quantity of rRNA in a total RNA sample can vary significantly between samples. An input of 100 ng – 1 µg of total RNA is recommended to ensure that sufficient rRNA-depleted RNA is available for downstream library preparation.
- RNA in volumes >10 µL should be concentrated to 10 µL prior to use by 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 12 µL in RNAse-free water is recommended to ensure that 10 µL is available for use in this protocol.
- It is recommended to assess the quality and size distribution of the input RNA prior to rRNA depletion by an electrophoretic method (e.g., Agilent Bioanalyzer RNA assay).
- The quality of RNA extracted from formalin-fixed paraffin embedded (FFPE) tissue can be highly variable due to the damaging nature of the formalin fixation process where crosslinking, chemical modification, and fragmentation can occur. Library construction results may vary depending on the input amount and quality of the RNA. Inputting more RNA (with a maximum of 1 µg) may salvage library construction with particularly difficult FFPE samples.

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.

RNA Fragmentation

- RNA is fragmented by incubating at a high temperature in the presence of magnesium before carrying out 1st strand cDNA synthesis.
- Fragmentation conditions given in the **Library Construction Protocol** should be used as a guideline. Fragmentation times may require adjustment based upon the quality and size distribution of the input RNA. It is recommended that a non-precious, representative sample of RNA be evaluated for the optimal fragmentation conditions.
- For intact RNA such as that extracted from fresh/frozen tissue, longer fragmentation is required at higher temperatures. For degraded or fragmented RNA (e.g., from older samples or FFPE tissue), use a lower temperature and/or shorter time.

Safe Stopping Points

The library construction process from rRNA depletion through library amplification can be performed in 10 – 12 hrs, depending on the number of samples being processed and experience. If necessary, the protocol may be paused safely at any of the following steps:

- After **2nd Strand Synthesis and Marking Cleanup** (steps 9.1 – 9.13), resuspend the washed beads in 15 µL of 1X A-Tailing Buffer, and store at 2°C to 8°C for ≤24 hrs.
- After **1st Post-ligation Cleanup** (steps 12.1 – 12.15), store the resuspended beads at 2°C to 8°C for ≤24 hrs.
- After **2nd Post-ligation Cleanup** (steps 13.1 – 13.17), 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 – 9.

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 either KAPA Pure Beads or Agencourt AMPure XP (Beckman Coulter). Solutions and conditions for nucleic acid 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 nucleic acid recovery, it is critical that the nucleic acid and KAPA Pure Beads are thoroughly mixed*** (by vortexing or extensive up-and-down pipetting) before the nucleic acid 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 and RNA. 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 at -15°C to -25°C for long-term storage. 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 RNA-Seq Kit with RiboErase (HMR). 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 **sequencing.roche.com/support**.
- Adapter concentration affects ligation efficiency, as well as adapter and adapter-dimer carry-over during post-ligation cleanups. The optimal adapter concentration for your workflow represents a compromise between the above factors and cost.
- 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 highly degraded RNA inputs, such as RNA extracted from FFPE tissue or 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 x 10^{-7} errors/base, equivalent to 1 error per 3.5 x 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 optimal amplification efficiency and avoid primer depletion, it is critical to use an optimal concentration of high quality primers. Primers should be used at a final concentration of 0.5 – 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 14)—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.

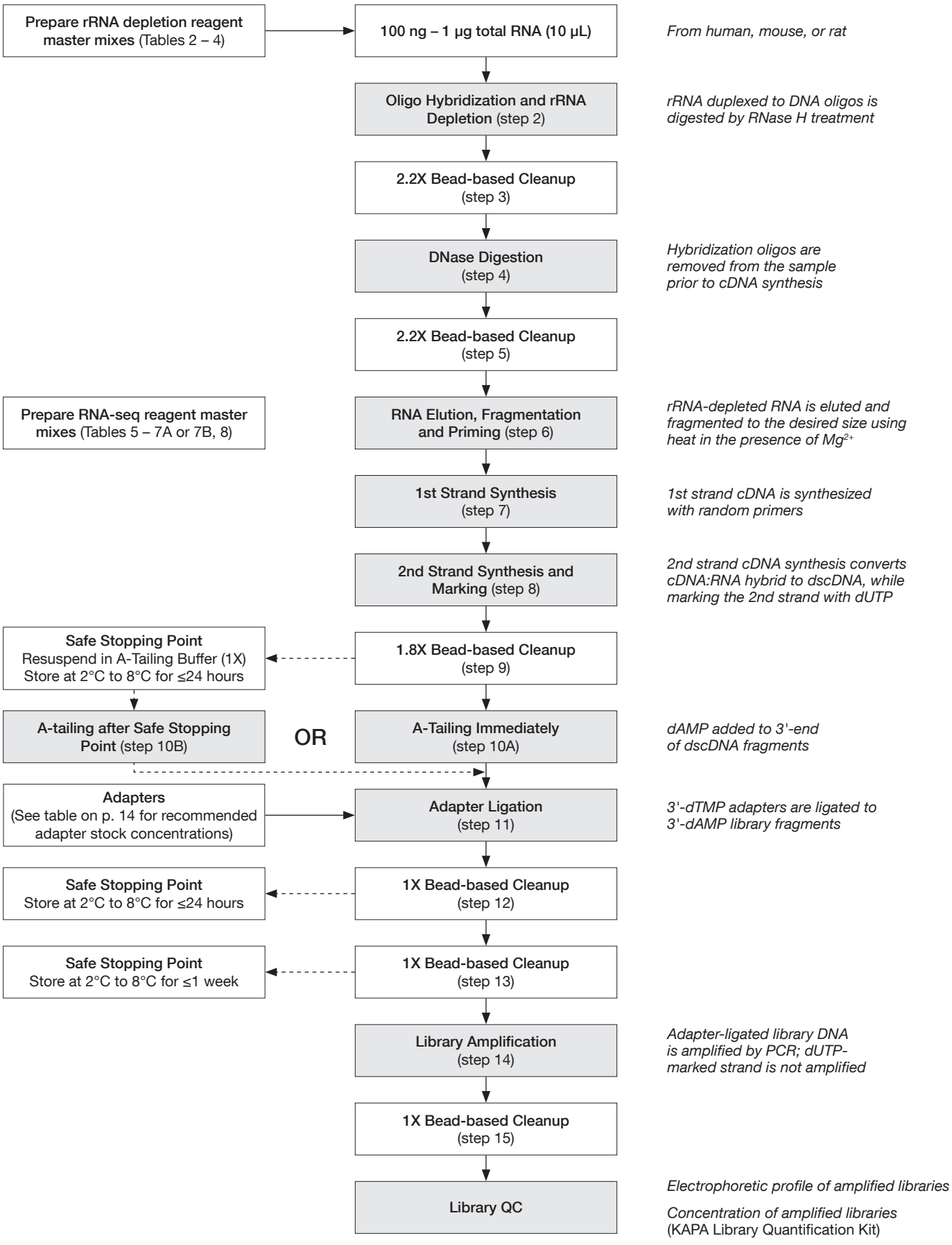
Table 1. Recommended library amplification cycles

Quantity of starting material	Number of cycles
100 – 250 ng	12 – 16
251 – 500 ng	10 – 13
501 – 1000 ng	8 – 11

Evaluating the Success of Library Construction

- Your specific library construction workflow should be tailored and optimized to yield a sufficient number of adapter-ligated molecules of the desired size distribution for sequencing, QC, and archiving purposes.
- The size distribution of final libraries should be confirmed with an electrophoretic method. A LabChip GX, GXII, or GX Touch (PerkinElmer), Bioanalyzer or TapeStation (Agilent Technologies), Fragment Analyzer (Advanced Analytics), or similar instrument is recommended over conventional gels.
- KAPA Library Quantification Kits for Illumina platforms are recommended for qPCR-based quantification of libraries generated with the KAPA Stranded RNA-Seq Kits with RiboErase (HMR). 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 10 – 12 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 RNA-Seq Kit with RiboErase (HMR). 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 – 9.

Volumes of additional reagents required for the KAPA Stranded RNA-Seq Kit with RiboErase (HMR) protocol are listed in Table 10.

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 of 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 this safe stopping point are provided in Table 7B.

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

Table 2. Oligo hybridization

Component:	1 library	8 libraries *Inc. 10% excess*	24 libraries *Inc. 10% excess*	96 libraries *Inc. 10% excess*
Hybridization master mix				
Hybridization Buffer	4 µL	35.2 µL	106 µL	423 µL
Hybridization Oligos (HMR)	4 µL	35.2 µL	106 µL	423 µL
RNase-free water	2 µL	17.6 µL	53 µL	211 µL
Total master mix volume:	**10 µL**	**88 µL**	**265 µL**	**1057 µL**
Final reaction composition:	**Per reaction**			
Hybridization master mix	10 µL			
Total RNA	10 µL			
Total reaction volume:	**20 µL**			

Table 3. rRNA depletion

Component:	1 library	8 libraries *Inc. 10% excess*	24 libraries *Inc. 10% excess*	96 libraries *Inc. 10% excess*
Depletion master mix				
Depletion Buffer	3 µL	26.4 µL	80 µL	317 µL
RNase H	2 µL	17.6 µL	53 µL	211 µL
Total master mix volume:	**5 µL**	**44.0 µL**	**133 µL**	**528 µL**
Final reaction composition:	**Per reaction**			
Depletion master mix	5 µL			
Total RNA hybridized to oligos	20 µL			
Total reaction volume:	**25 µL**			

Table 4. DNase digestion

Component:	1 library	8 libraries *Inc. 10% excess*	24 libraries *Inc. 10% excess*	96 libraries *Inc. 10% excess*
DNase digestion master mix				
DNase Buffer	2.2 µL	19.4 µL	58 µL	232 µL
DNase	2 µL	17.6 µL	53 µL	211 µL
RNase-free water	17.8 µL	157 µL	470 µL	1880 µL
Total master mix volume:	**22 µL**	**194 µL**	**581 µL**	**2323 µL**
Resuspend beads in a volume of:	**22 µL**			

Table 5. 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 6. 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 7A. 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 7B. 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 8. 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
T4 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 (140 – 280 nM, as appropriate)	5 µL			
Total reaction volume:	**70 µL**			

Table 9. 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 10. 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)				
rRNA depletion cleanups	99 µL	880 µL	2.6 mL	10.5 mL
2nd strand synthesis and marking cleanup	108 µL	950 µL	2.9 mL	11.4 mL
Library amplification cleanup	50 µL	440 µL	1.3 mL	5.3 mL
Total volume required:	**257 µL**	**2270 µL**	**6.8 mL**	**27.2 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)				
rRNA depletion cleanups	0.8 mL	7.0 mL	21.1 mL	84.5 mL
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:	**2.4 mL**	**21.0 mL**	**63.5 mL**	**253.3 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. Oligo Hybridization and rRNA Depletion

This protocol requires 100 ng – 1 µg of total RNA, in 10 µL of RNase-free water.

Ensure that the hybridization master mix (Table 2) and the depletion master mix (Table 3) are prepared and kept at room temperature before use.

2.1 Program a thermocycler as follows:

Step	Temp.	Duration
Hybridization	95°C	2 min
Ramp down to 45°C at -0.1°C/s		
PAUSE	45°C	∞
Depletion	45°C	30 min
HOLD	4°C	∞

2.2 Assemble rRNA hybridization reactions as follows:

Component	Volume
Total RNA in water	10 µL
Hybridization master mix at room temperature (Table 2)	10 µL
Total volume:	**20 µL**

2.3 Place samples in the pre-programmed thermocycler and execute the program.

2.4 Ensure the depletion master mix containing RNase H is added while the samples are kept at 45°C in a thermocycler. When the program reaches the pause step at 45°C, add the following to each 20 µL hybridization reaction and mix thoroughly by pipetting up and down multiple times.

Component	Volume
Depletion master mix at room temperature (Table 3)	5 µL
Total volume:	**25 µL**

2.5 Resume the cycling program to continue with the depletion step (45°C for 30 min).

2.6 Proceed immediately to **rRNA Depletion Cleanup** (step 3).

3. rRNA Depletion Cleanup

3.1 Perform a 2.2X bead-based cleanup by combining the following:

Component	Volume
rRNA-depleted RNA	25 µL
KAPA Pure Beads	55 µL
Total volume:	**80 µL**

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

3.3 Incubate the plate/tube(s) at room temperature for 5 min to bind the RNA to the beads.

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

3.5 Carefully remove and discard 75 µL of supernatant.

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

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

3.8 Carefully remove and discard the ethanol.

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

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

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

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

4. DNase Digestion

To remove the hybridization oligo-nucleotides from the ribosomal depleted RNA, the sample is incubated with DNase. ***Ensure that the DNase digestion master mix (Table 4) is prepared and kept at room temperature.***

4.1 Assemble DNase digestion reactions as follows:

Component	Volume
Beads with ribosomal-depleted RNA	–
DNase digestion master mix at room temperature (Table 4)	22 µL
Total volume:	**22 µL**

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

4.3 Incubate the plate/tube(s) at room temperature for 3 min to elute the RNA off the beads.

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

4.5 Carefully transfer 20 µL of supernatant into a new plate/tube(s). Discard the plate/tube(s) with beads.

4.6 Incubate the plate/tube(s) with supernatant using the following protocol:

Step	Temp.	Duration
DNase digestion	37°C	30 min
HOLD	4°C	∞

4.7 Proceed immediately to **DNase Digestion Cleanup** (step 5).

5. DNase Digestion Cleanup

5.1 Perform a 2.2X bead-based cleanup by combining the following:

Component	Volume
DNase-treated RNA	20 µL
KAPA Pure Beads	44 µL
Total volume:	**64 µL**

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

5.3 Incubate the plate/tube(s) at room temperature for 5 min to bind the RNA to the beads.

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

5.5 Carefully remove and discard 60 µL of supernatant.

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

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

5.8 Carefully remove and discard the ethanol.

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

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

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

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

6. RNA Elution, Fragmentation, and Priming

RNA depleted of rRNA is eluted from beads in Fragment, Prime and Elute Buffer (1X) and fragmented to the desired size by incubation at high temperature.

6.1 Prepare the required volume of Fragment, Prime and Elute Buffer (1X) by combining the following at room temperature:

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

6.2 Thoroughly resuspend the beads with purified, DNase-treated RNA in 22 µL of Fragment, Prime and Elute Buffer (1X) by pipetting up and down multiple times.

6.3 Incubate the plate/tube(s) at room temperature for 3 min to elute RNA off 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 transfer 20 µL of supernatant into a new plate/tube(s). Discard the plate/tube(s) with beads.

6.6 Place the plate/tube(s) in a thermocycler and carry out the fragmentation and priming program as follows:

Input RNA type	Desired mean library insert size (bp)	Fragmentation
Intact	100 – 200	8 min at 94°C
	200 – 300	6 min at 94°C
	300 – 400	6 min at 85°C
Partially degraded	100 – 300	1 – 6 min at 85°C
Degraded	100 – 200	1 min at 65°C

6.7 Place the plate/tube(s) on ice and proceed immediately to **1st Strand Synthesis** (step 7).

7. 1st Strand Synthesis

7.1 On ice, assemble the 1st strand synthesis reaction as follows:

Component	Volume
Fragmented, primed RNA	20 µL
1st strand synthesis master mix (Table 5)	10 µL
Total volume:	**30 µL**

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

7.3 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	∞

7.4 Place the plate/tube(s) on ice and proceed immediately to **2nd Strand Synthesis and Marking** (step 8).

8. 2nd Strand Synthesis and Marking

8.1 Assemble the 2nd strand synthesis and marking reaction as follows:

Component	Volume
1st strand cDNA	30 µL
2nd strand synthesis and marking master mix (Table 6)	30 µL
Total volume:	**60 µL**

8.2 Mix thoroughly by gently pipetting the reaction up and down several times.

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

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

8.4 Proceed immediately to **2nd Strand Synthesis and Marking Cleanup** (step 9).

9. 2nd Strand Synthesis and Marking Cleanup

9.1 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**

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 160 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 Proceed immediately to **A-tailing Immediately** (step 10A), or follow the Safe Stopping Point instructions.

SAFE STOPPING POINT

Resuspend the beads in 15 µL A-Tailing Buffer (1X) (Table 7B), 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 10B).

10. 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 10A) or **A-tailing after Safe Stopping Point** (step 10B).

10A. A-tailing Immediately

10A.1 Assemble the A-tailing reaction as follows:

Component	Volume
Beads with dscDNA	–
A-tailing master mix (Table 7A)	30 µL
Total volume:	**30 µL**

10A.2 Mix thoroughly by pipetting up and down several times.

10A.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	∞

10A.4 Proceed immediately to **Adapter Ligation** (step 11).

10B. A-tailing after Safe Stopping Point

10B.1 To resume library preparation, combine the following reagents to perform A-tailing:

Component	Volume
Beads with dscDNA (in A-Tailing Buffer (1X), Table 7B)	15 µL
A-tailing master mix after safe stopping point (Table 7B)	15 µL
Total volume:	**30 µL**

10B.2 Mix thoroughly by pipetting up and down several times.

10B.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	∞

10B.4 Proceed immediately to **Adapter Ligation** (step 11).

11. Adapter Ligation

11.1 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	210 nM	15 nM
501 – 1000 ng	280 nM	20 nM

11.2 Set up the adapter ligation reactions as follows:

Component	Volume
Beads with A-tailed DNA	30 µL
Adapter ligation master mix (Table 8)	35 µL
Diluted adapter stock	5 µL
Total volume:	**70 µL**

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

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

11.5 Proceed immediately to **1st Post-ligation Cleanup** (step 12).

12. 1st Post-ligation Cleanup

12.1 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**

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 135 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 beads in 50 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.

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 13).

13. 2nd Post-ligation Cleanup

13.1 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**

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

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

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

13.5 Carefully remove and discard 95 µL of supernatant.

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

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

13.8 Carefully remove and discard the ethanol.

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

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

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

13.12 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.***

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

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

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

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

13.17 Transfer 20 µL of the clear supernatant to a new plate/tube(s) and proceed to **Library Amplification** (step 14).

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 14).

14. Library Amplification

14.1 Assemble each library amplification reaction as follows:

Component	Volume
Purified, adapter-ligated DNA	20 µL
Library amplification master mix (Table 9)	30 µL
Total volume:	**50 µL**

14.2 Mix well by pipetting up and down several times.

14.3 Amplify the library using the following thermocycling 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.

14.4 Proceed immediately to **Library Amplification Cleanup** (step 15).

15. Library Amplification Cleanup

15.1 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**

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

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

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

15.5 Carefully remove and discard 95 µL of supernatant.

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

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

15.8 Carefully remove and discard the ethanol.

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

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

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

15.12 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.***

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

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

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

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

15.17 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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