KAPABIOSYSTEMS

Technical Data Sheet

KAPA mRNA HyperPrep Kit
Illumina® Platforms

KR1352 – v5.17

This Technical Data Sheet provides product information and a detailed protocol for the KAPA mRNA HyperPrep Kit for Illumina Platforms.

The document applies to KAPA mRNA HyperPrep Kits (08098115702 and 08098123702) and KAPA mRNA Capture Kits (07962231001 and 07962240001).

Contents

Kapa/*Roche* Kit Codes and Components		
KK8580 *08098115702* 24 libraries = **KK8542** *08105936001* + **KK8543** *08105880001* + **KK8440** *07962231001*	mRNA Capture Beads mRNA Bead Binding Buffer mRNA Bead Wash Buffer RNase-free Water Fragment, Prime and Elute Buffer (2X) 1st Strand Synthesis Buffer KAPA Script 2nd Strand Marking Buffer 2nd Strand Synthesis & A-Tailing Enzyme Mix Ligation Buffer DNA Ligase PEG/NaCl Solution KAPA Pure Beads Library Amplification Primer Mix (10X) KAPA HiFi HotStart ReadyMix (2X)	1.3 mL 3.9 mL 9.6 mL 6.5 mL 300 µL 300 µL 25 µL 780 µL 50 µL 1 mL 280 µL 1 mL 3.2 mL 138 µL 690 µL
KK8581 *08098123702* 96 libraries = **KK8544** *08105952001* + **KK8545** *08105901001* + **KK8441** *07962240001*	mRNA Capture Beads mRNA Bead Binding Buffer mRNA Bead Wash Buffer RNase-free Water Fragment, Prime and Elute Buffer (2X) 1st Strand Synthesis Buffer KAPA Script 2nd Strand Marking Buffer 2nd Strand Synthesis & A-Tailing Enzyme Mix Ligation Buffer DNA Ligase PEG/NaCl Solution KAPA Pure Beads (3 bottles) Library Amplification Primer Mix (10X) KAPA HiFi HotStart ReadyMix (2X)	5.1 mL 15.3 mL 40 mL 25 mL 1.4 mL 1.4 mL 130 µL 3.8 mL 250 µL 5 mL 1.26 mL 5 mL 15 mL 600 µL 3 mL
KK8440 *07962231001* 24 libraries	mRNA Capture Beads mRNA Bead Binding Buffer mRNA Bead Wash Buffer RNase-free Water	1.2 mL 3.6 mL 9.6 mL 6.5 mL
KK8441 *07962240001* 96 libraries	mRNA Capture Beads mRNA Bead Binding Buffer mRNA Bead Wash Buffer RNase-free Water	4.8 mL 14.4 mL 40 mL 25 mL

Quick Notes

- Rapid and easily automatable protocol enables stranded library construction in approximately 5.5 hrs.
- This protocol is suitable for the construction of high-quality libraries from 50 ng – 1 µg of intact total RNA.
- Accurate strand origin information maintained using dUTP incorporation during second strand synthesis.
- To prevent foaming, avoid shaking or high-speed vortexing of mRNA capture beads.
- This kit contains KAPA Pure Beads for reaction cleanups, along with all reagents needed for library construction and high-efficiency, low-bias library amplification, except for adapters. KAPA Adapters are sold separately.
- Not compatible with small RNAs <100 bp in length.

Product Description

The KAPA mRNA HyperPrep Kit for Illumina sequencing contains all of the buffers and enzymes required for poly(A) mRNA capture and the rapid construction of stranded mRNA-Seq libraries from 50 ng – 1 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. combined 2nd strand synthesis and A-tailing, which converts the cDNA:RNA hybrid to double-stranded cDNA (dscDNA), incorporates dUTP into the second cDNA strand, and adds dAMP to the 3' ends of the resulting dscDNA;
5. adapter ligation, where dsDNA adapters with 3' dTMP overhangs are ligated to library insert fragments; and
6. 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 KAPA Pure Beads for reaction cleanups, along with all of the enzymes and buffers required for mRNA capture, cDNA synthesis, library construction and amplification, but does not include RNA or adapters. 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 has been designed for low-bias, high-fidelity PCR, and is the polymerase of choice for NGS library amplification[1,2,3,4]. The KAPA mRNA HyperPrep Kit 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 mRNA HyperPrep Kit is designed for both manual and automated NGS library construction from 50 ng – 1 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; and
- characterization of polyadenylated RNAs.

This kit is not compatible with small RNAs <100 bp in length.

Product Specifications

Shipping and Storage

KAPA mRNA HyperPrep Kits are supplied in multiple boxes:

Contents	Storage upon receipt
mRNA capture beads and buffers	2°C to 8°C
cDNA synthesis and library preparation reagents	-15°C to -25°C
KAPA Pure Beads	2°C to 8°C

Boxes containing KAPA Pure Beads and mRNA capture beads and buffers are shipped on dry ice or ice packs, depending on the destination country. ***Upon receipt, store KAPA Pure Beads and mRNA capture beads and buffers at 2°C to 8°C.*** Enzymes and buffers for cDNA synthesis and library preparation are shipped on dry ice or ice packs, depending on the destination country. These components are temperature sensitive, and appropriate care should be taken during storage. ***Upon receipt, store the enzymes and buffers for cDNA synthesis and library preparation at -15°C to -25°C*** in a constant-temperature freezer. KAPA Pure Beads, 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

Reagents for mRNA capture and KAPA Pure Beads ***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 KAPA Pure Beads and 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 ***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 reaction components and master mixes on ice whenever possible during handling and preparation.

The 1st Strand Synthesis Buffer, KAPA Pure Beads, 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 (2X) is fully thawed and thoroughly mixed before use.

The 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

- The protocol has been validated for library construction from 50 ng – 1 µg of purified, 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 50 ng – 1 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 beads, 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, elute in 55 µL of RNase-free water to ensure that 50 µL is available for use with 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* beads, 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 bead 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 microtube 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 (2X) 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 polyadenylated 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.
- For fragmentation optimization beyond what is provided in the **Library Construction Protocol**, please refer to **Appendix: Library Size Distribution Optimization** (p. 15).

Safe Stopping Points

The library construction process from mRNA capture through library amplification can be performed in approximately 5.5 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** (step 2), the resuspended beads may be stored at 2°C to 8°C for ≤24 hrs in 22 µL of Fragment, Prime and Elute Buffer (2X).
- After **1st Post-ligation Cleanup** (step 7), store the resuspended beads at 2°C to 8°C for ≤24 hrs.
- After **2nd Post-ligation Cleanup** (step 8), 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 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 – 5.

Libraries may be prepared in standard reaction vessels, including 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 the workflow, consider compatibility with:

- the magnet used during KAPA Pure 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 KAPA Pure Beads. 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 storage and handling recommendations for KAPA Pure Beads. 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 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 to completely capture beads varies according to the reaction vessel and magnet used. It is important not to discard or transfer any beads with the removal of the supernatant. Capture times should be optimized accordingly.
- The volumes of 80% ethanol for the bead washes may be adjusted to accommodate smaller reaction vessels and/or limiting 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 may 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 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 mRNA HyperPrep 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. Truncated adapter designs, where cluster generation sequences are added during amplification instead of ligation, may require modified post-ligation cleanup conditions. For assistance with adapter compatibility, ordering, and duplexing, please visit **sequencing.roche.com/support**.
- Adapter concentration affects ligation efficiency, as well as adapter and adapter-dimer carryover during post-ligation cleanups. The optimal adapter concentration for the 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 (50 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 (2X), is an antibody-based hot start formulation of KAPA HiFi DNA Polymerase, a novel B-family DNA polymerase engineered for increased processivity and high fidelity. KAPA HiFi HotStart DNA Polymerase has 5'→3' polymerase and 3'→5' exonuclease (proofreading) activities, but no 5'→3' exonuclease activity. The strong 3'→5' exonuclease activity results in superior accuracy during DNA amplification. The error rate of KAPA HiFi HotStart DNA Polymerase is 2.8×10^{-7} errors/base, equivalent to 1 error per 3.5×10^{6} nucleotides incorporated.
- Library Amplification Primer Mix (10X) is designed to eliminate or delay primer depletion during library amplification reactions performed with KAPA HiFi HotStart ReadyMix (2X). 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 primers may be used in combination with custom adapters. For guidelines on the formulation of user-supplied library amplification primers, please contact Technical Support at **sequencing.roche.com/support**.
- 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 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 4°C for short-term use, or as single-use aliquots at -20°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 the 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 electrophoretic analysis of amplified libraries. However, they typically

comprise library molecules of the desired length, which are individualized during denaturation prior to cluster amplification. 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 quantification 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 9)—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.
- 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, presence of adapter-dimer, and the desired yield post-amplification. Quantification of material after the second post-ligation cleanup using a qPCR assay, such as the KAPA Library Quantification Kit, can help to determine the number of amplification cycles required for a specific sample type or application.

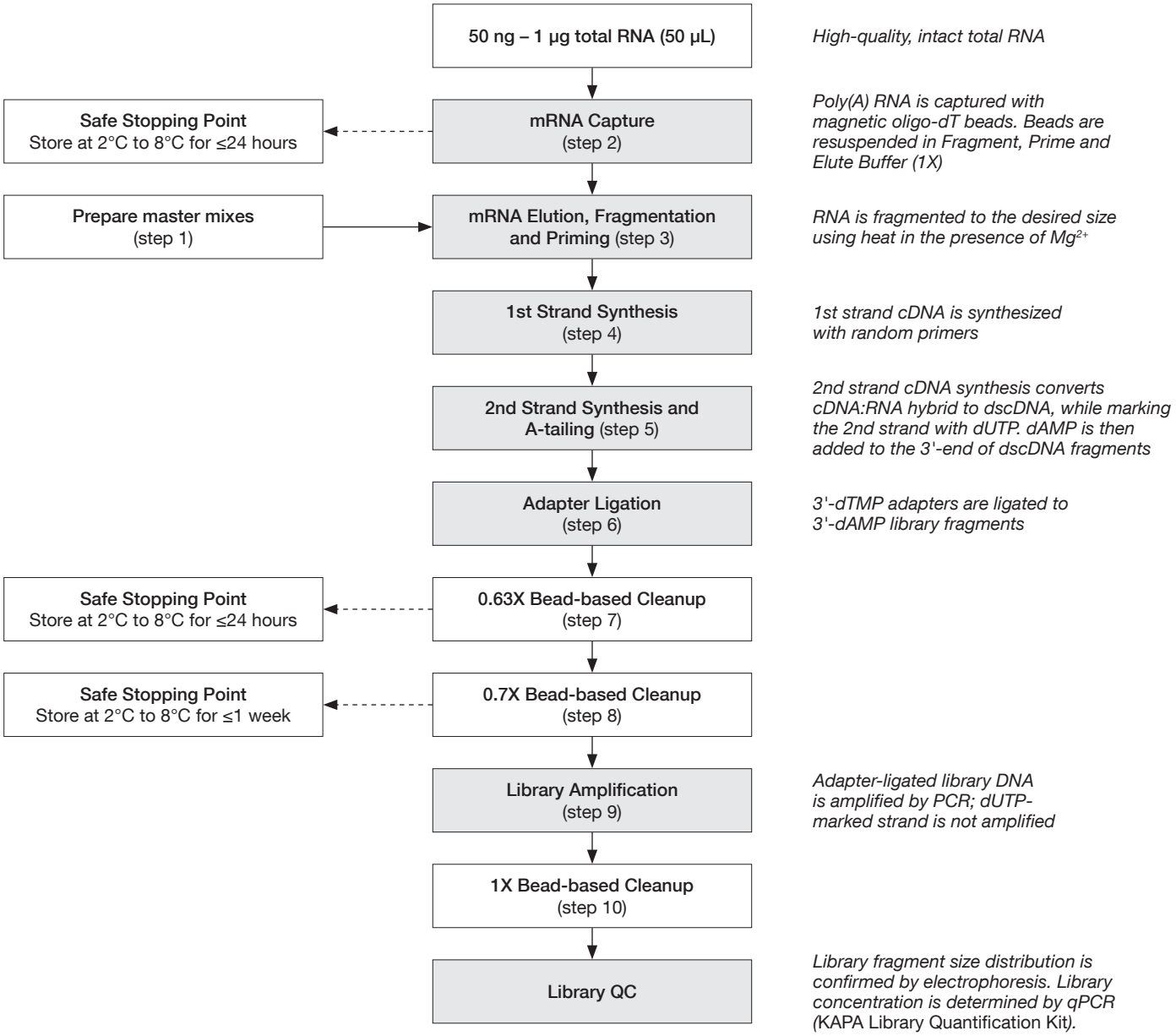
Table 1. Recommended library amplification cycles

Quantity of starting material	Number of cycles
50 – 100 ng	13 – 16
101 – 250 ng	11 – 14
251 – 500 ng	9 – 12
501 – 1000 ng	7 – 10

Evaluating the Success of Library Construction

- A specific library construction workflow should be tailored and optimized to yield a sufficient amount of adapter-ligated molecules of the desired size distribution for 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 Analytical Technologies) 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 mRNA HyperPrep Kit. 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 with minimal bias.

Process Workflow



Library Construction Protocol

1. Reagent Preparation

This protocol takes approximately 5.5 hrs to complete. Ideally, master mixes for the various steps in the process should be prepared as required.

For maximum stability and shelf-life, the enzymes and reaction buffers are supplied separately in the KAPA mRNA HyperPrep Kit.

For a streamlined protocol, a reagent master mix with a minimum of 10% excess is prepared for each of these enzymatic steps, as outlined in Tables 2 – 5. Volumes of additional reagents required for the KAPA mRNA HyperPrep Kit protocol are listed in Table 6.

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*	N libraries *Inc. 20% excess*
1st strand synthesis master mix:					
1st Strand Synthesis Buffer	11 µL	88 µL	264 µL	1056 µL	N*11 µL
KAPA Script	1 µL	8 µL	24 µL	96 µL	N*1 µL
Total master mix volume:	**12 µL**	**96 µL**	**288 µL**	**1152 µL**	**N*12 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 A-tailing

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

Table 4. Adapter ligation

Component	1 library *Inc. 10% excess*	8 libraries *Inc. 10% excess*	24 libraries *Inc. 10% excess*	96 libraries *Inc. 10% excess*	N libraries *Inc. 10% excess*
Adapter ligation master mix:					
Ligation Buffer	40 µL	320 µL	960 µL	3840 µL	N*40 µL
DNA Ligase	10 µL	80 µL	240 µL	960 µL	N*10 µL
Total master mix volume:	**50 µL**	**400 µL**	**1200 µL**	**4800 µL**	**N*50 µL**
Final reaction composition:	**Per reaction**				
Adapter ligation master mix	45 µL				
A-tailed dscDNA	60 µL				
Adapter, 1.5 µM or 7 µM, see step 6.1	5 µL				
Total reaction volume:	**110 µL**				

Table 5. Library amplification

Component	1 library *No excess*	8 libraries *Inc. 10% excess*	24 libraries *Inc. 10% excess*	96 libraries *Inc. 10% excess*	N libraries *Inc. 10% excess*
Library amplification master mix:					
KAPA HiFi HotStart ReadyMix (2X)	25 µL	220 µL	660 µL	2640 µL	N*27.5 µL
Library Amplification Primer Mix (10X)	5 µL	44 µL	132 µL	528 µL	N*5.5 µL
Total master mix volume:	**30 µL**	**264 µL**	**792 µL**	**3168 µL**	**N*33 µL**
Final reaction composition:	**Per reaction**				
Library amplification master mix	30 µL				
Adapter-ligated DNA	20 µL				
Total reaction volume:	**50 µL**				

Table 6. Volumes of additional reagents required

Component	1 library *No excess*	8 libraries *Inc. ≥10% excess*	24 libraries *Inc. ≥10% excess*	96 libraries *Inc. ≥10% excess*	N libraries *Inc. ≥10% excess*
KAPA Pure Beads (provided in kit):					
1st post-ligation cleanup	70 µL	620 µL	1.9 mL	7.4 mL	N*77 µL
Library amplification cleanup	50 µL	440 µL	1.4 mL	5.3 mL	N*55 µL
Total volume required:	**120 µL**	**1060 µL**	**3.3 mL**	**12.7 mL**	**N*132 µL**
Component	**1 library** *No excess*	**8 libraries** *Inc. ≥10% excess*	**24 libraries** *Inc. ≥10% excess*	**96 libraries** *Inc. ≥10% excess*	**N libraries** *Inc. ≥10% excess*
PEG/NaCl Solution (provided in kit):					
2nd post-ligation cleanup	35 µL	310 µL	930 µL	3.7 mL	N*38.5 µL
Total volume required:	**35 µL**	**310 µL**	**930 µL**	**3.7 mL**	**N*38.5 µL**
Component	**1 library** *No excess*	**8 libraries** *Inc. ≥10% excess*	**24 libraries** *Inc. ≥10% excess*	**96 libraries** *Inc. ≥10% excess*	**N libraries** *Inc. ≥10% excess*
80% ethanol (freshly prepared; not supplied):					
1st post-ligation cleanup	0.4 mL	3.6 mL	10.6 mL	42.3 mL	N*0.5 mL
2nd post-ligation cleanup	0.4 mL	3.6 mL	10.6 mL	42.3 mL	N*0.5 mL
Library amplification cleanup	0.4 mL	3.6 mL	10.6 mL	42.3 mL	N*0.5 mL
Total volume required:	**1.2 mL**	**10.8 mL**	**31.8 mL**	**126.9 mL**	**N*1.5 mL**
Component	**1 library** *No excess*	**8 libraries** *Inc. ≥10% excess*	**24 libraries** *Inc. ≥10% excess*	**96 libraries** *Inc. ≥10% excess*	**N 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	1.4 mL	5.3 mL	N*55 µL
2nd post-ligation cleanup	22 µL	200 µL	0.6 mL	2.4 mL	N*25 µL
Library amplification cleanup	22 µL	200 µL	0.6 mL	2.4 mL	N*25 µL
Total volume required:	**94 µL**	**840 µL**	**2.6 mL**	**10.1 mL**	**N*105 µL**

2. mRNA Capture

This protocol requires 50 ng – 1 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 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 levels in the captured mRNA.

Before starting, equilibrate mRNA Capture Beads, mRNA Bead Binding Buffer, mRNA Bead Wash Buffer and Fragment, Prime and Elute Buffer (2X) 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. 4) 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 (1260 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** 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 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.

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
200 – 300	6 min at 94°C
300 – 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 1st 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 A-tailing** (step 5).

5. 2nd Strand Synthesis and A-tailing

5.1 On ice, assemble the 2nd strand synthesis and A-tailing reaction as follows:

Component	Volume
1st strand synthesis product	30 µL
2nd strand synthesis and A-tailing 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	16°C	30 min
A-tailing	62°C	10 min
HOLD	4°C	∞

5.4 Place the plate/tube(s) on ice and proceed immediately to **Adapter Ligation** (step 6).

6. Adapter Ligation

6.1 Dilute adapters in preparation for ligation, targeting the following concentrations:

Quantity of starting material	Adapter stock concentration
50 – 499 ng	1.5 µM
500 – 1000 ng	7 µM

6.2 On ice, set up the adapter ligation reaction as follows:

Component	Volume
2nd strand synthesis product	60 µL
Adapter ligation master mix (Table 4)	45 µL
Diluted adapter stock	5 µL
Total volume:	**110 µL**

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

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

6.5 Proceed immediately to **1st Post-ligation Cleanup** (step 7).

7. 1st Post-ligation Cleanup

7.1 Perform a 0.63X bead-based cleanup by combining the following:

Component	Volume
Adapter-ligated DNA	110 µL
KAPA Pure Beads	70 µL
Total volume:	**180 µL**

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

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

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

7.5 Carefully remove and discard 175 µL of supernatant.

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

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

7.8 Carefully remove and discard the ethanol.

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

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

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

7.12 Dry the beads 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.***

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

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

7.15 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 8).

8. 2nd Post-ligation Cleanup

8.1 Perform a 0.7X bead-based cleanup by combining the following:

Component	Volume
Beads with purified, adapter-ligated DNA	50 µL
PEG/NaCl Solution	35 µL
Total volume:	**85 µL**

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

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

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

8.5 Carefully remove and discard 80 µL of supernatant.

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

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

8.8 Carefully remove and discard the ethanol.

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

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

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

8.12 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.***

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

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

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

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

8.17 Transfer 20 µL of the clear supernatant to a new plate/tube(s) and proceed to **Library Amplification** (step 9).

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 9).

9. Library Amplification

9.1 Assemble each library amplification reaction as follows:

Component	Volume
Purified, adapter-ligated DNA	20 µL
Library amplification master mix (Table 5)	30 µL
Total volume:	**50 µL**

9.2 Mix well by pipetting up and down several times.

9.3 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	1 min	1
HOLD	4°C	∞	1

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

9.4 Proceed immediately to **Library Amplification Cleanup** (step 10).

10. Library Amplification Cleanup

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

Component	Volume
Adapter-ligated DNA	50 µL
KAPA Pure Beads	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 Thoroughly resuspend the dried beads in 22 µL of 10 mM Tris-HCl (pH 8.0 – 8.5).

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

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

10.16 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.

Appendix: Library Size Distribution Optimization

RNA Fragmentation

The KAPA mRNA HyperPrep Kit offers a tunable RNA fragmentation module where RNA is fragmented by incubating at a high temperature in the presence of magnesium. Final library insert size can be optimized for specific applications by varying both incubation time and temperature. Generally:

- higher temperatures and/or longer incubation times result in shorter, narrower distributions; and
- lower temperatures and/or shorter incubation times result in longer, broader distributions.

Figure 1 and Table 7 can be used as a starting point for the optimization of final library distributions beyond what is provided within the **Library Construction Protocol**. It is recommended that a non-precious, representative RNA sample be used for fragmentation condition optimization. For the following figures and tables, final libraries were generated using 100 ng of high-quality Universal Human Reference (UHR) RNA with the KAPA mRNA HyperPrep Kit.

The approximate mean and mode for the distributions shown in Figure 1 are summarized in Table 7. Figure 2 visually depicts these metrics for a sample library.

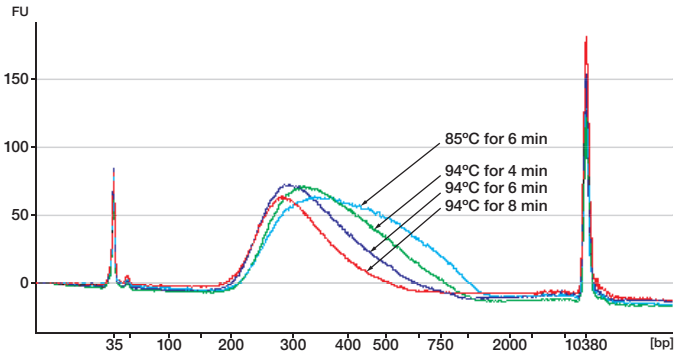


Figure 1. Final library distributions demonstrating fragmentation tunability

Higher temperatures and longer incubation times resulted in shorter, narrower final library distributions. Libraries were constructed using 100 ng of high-quality UHR RNA and various fragmentation conditions. Note that results may differ with other sample sources. Electropherograms were generated with an Agilent High Sensitivity DNA Kit.

Table 7. Approximate mean and mode final library sizes (bp) for each fragmentation condition assessed

Fragmentation	Final library size (bp)	
	Mean	Mode
94°C for 8 min	~320	~290
94°C for 6 min	~350	~300
94°C for 4 min	~390	~330
94°C for 2 min	~470	~380
85°C for 6 min	~430	~330
85°C for 4 min	~490	~450

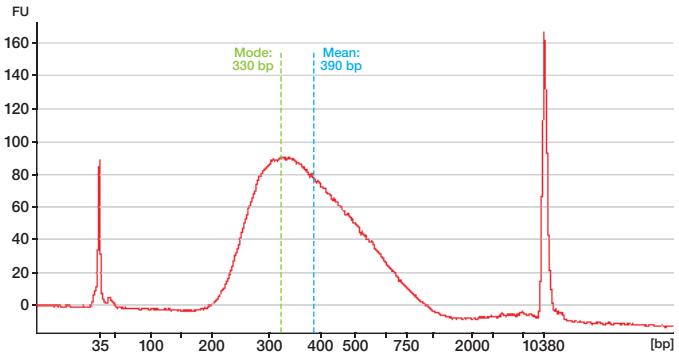


Figure 2. Visual depiction of the mean and mode distribution metrics

For a final library generated using 100 ng UHR fragmented at 94°C for 4 minutes, the mode is the highest peak in the library (~330 bp), while the mean is the numerical average across all molecular lengths in the library (~390 bp). In this example, the mean of the library is calculated across the range of 190 to 1600 bp. The higher molecular weight shoulder of the distribution results in the mean being larger than the mode.

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