KAPABIOSYSTEMS

Technical Data Sheet

KAPA Stranded RNA-Seq Library Preparation Kit

Illumina® platforms

KR0934 – v1.13

This Technical Data Sheet provides product information and a detailed protocol for the KAPA Stranded RNA-Seq Library Preparation Kit (Illumina® platforms), product codes KK8400 and KK8401.

Contents

Kit Codes and Components

KK8400 – 24 Libraries

KAPA Fragment, Prime and Elute Buffer (2X)	264 µl
KAPA 1st Strand Synthesis Buffer	264 µl
KAPA Script	25 µl
KAPA 2nd Strand Marking Buffer	750 µl
KAPA 2nd Strand Synthesis Enzyme Mix	50 µl
KAPA A-Tailing Buffer (10X)	80 µl
KAPA A-Tailing Enzyme	80 µl
KAPA Ligation Buffer (5X)	380 µl
KAPA DNA Ligase	135 µl
KAPA PEG/NaCl SPRI® Solution	5 ml
KAPA Library Amplification Primer Mix (10X)	138 µl
KAPA HiFi HotStart ReadyMix (2X)	690 µl

KK8401 – 96 libraries

KAPA Fragment, Prime and Elute Buffer (2X)	1.32 ml
KAPA 1st Strand Synthesis Buffer	1.32 ml
KAPA Script	120 µl
KAPA 2nd Strand Marking Buffer	3.72 ml
KAPA 2nd Strand Synthesis Enzyme Mix	240 µl
KAPA A-Tailing Buffer (10X)	650 µl
KAPA A-Tailing Enzyme	360 µl
KAPA Ligation Buffer (5X)	1.7 ml
KAPA DNA Ligase	600 µl
KAPA PEG/NaCl SPRI® Solution	30 ml
KAPA Library Amplification Primer Mix (10X)	600 µl
KAPA HiFi HotStart ReadyMix (2X)	3 ml

Quick Notes

- This protocol is suitable for 10 – 400 ng of poly(A)-enriched, or rRNA-depleted total input RNA, in a volume of 10 µl.
- Accurate strand origin information is retained in >99% of unique mapped reads.
- Adapters are not supplied with this kit, and can be obtained from any reputable oligonucleotide vendor.
- This protocol requires the use of Agencourt® AMPure® XP reagent (Beckman Coulter part number A63882), which is not included in the kit.
- PEG/NaCl SPRI® solution is provided for "with-bead" reaction cleanups.
- Separate buffer and enzyme mixes provide the best combination of product stability, convenience, and efficiency.
- KAPA HiFi HotStart ReadyMix is specifically designed to minimize the effects of amplification bias while maintaining industry leading fidelity.
- This protocol allows for the isolation and fragmentation of intact poly(A) RNA purified using oligo-(dT) capture beads (not supplied with the kit) prior to library preparation.

Product Description

The KAPA Stranded RNA-Seq Library Preparation Kit for Illumina® sequencing contains all of the buffers and enzymes required for the construction of libraries from 10 – 400 ng of rRNA-depleted or poly(A)-enriched RNA via the following steps:

1. Fragmentation using heat and magnesium.
2. 1st strand cDNA synthesis using random priming.
3. 2nd strand synthesis and marking, which converts the cDNA:RNA hybrid to double-stranded cDNA (dscDNA), and incorporates dUTP into the second cDNA strand.
4. A-tailing, to add dAMP to the 3'-ends of the dscDNA library fragments.
5. Adapter ligation, where dsDNA adapters with 3'-dTMP overhangs are ligated to A-tailed library insert fragments.
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.

Reaction buffers are supplied in convenient formats comprising all of the required reaction components except input RNA and adapters. Similarly, a single enzyme mixture is provided for each step of the library construction process, reducing the number of pipetting steps. There is no requirement to add water to the 1st strand synthesis and 2nd strand synthesis and marking master mixes. This minimizes the risk of RNase contamination, ensures consistent and homogenous reaction composition, and improves uniformity among replicate samples.

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] KAPA Stranded RNA-Seq Library Preparation Kits include KAPA HiFi HotStart ReadyMix (2X), and KAPA 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).

Product Applications

This KAPA Stranded RNA-Seq Library Preparation Kit for Illumina® is suitable for the construction of stranded RNA-Seq libraries from 10 – 400 ng of rRNA-depleted or poly(A)-enriched RNA.

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 Library Preparation Kits are shipped on dry ice or ice packs, depending on the destination country. Upon receipt, immediately store enzymes and reaction buffers at -20 °C in a constant-temperature freezer. The PEG/NaCl SPRI® Solution should be protected from light, and stored at -20 °C. For short-term use, the PEG/NaCl SPRI® Solution may be stored protected from light at 4 °C for up to 2 months. 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. KAPA HiFi HotStart ReadyMix (2X) may not freeze completely, even when stored at -20 °C. Nevertheless, always ensure that the KAPA HiFi HotStart ReadyMix is fully thawed and thoroughly mixed before use. PEG/NaCl SPRI® Solution does not freeze at -20 °C, but should be equilibrated to room temperature and mixed thoroughly before use.

Quality control

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

Important Parameters

Reaction setup

While this kit is intended for manual library construction, the protocol is designed to be automation-friendly in order to facilitate the transition to automation should throughput requirements grow over time. For this reason, and 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 5 – 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 3 – 7.

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 DNase-, RNase- and nuclease-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 SPRI® bead manipulations
- vortex mixers and centrifuges, where appropriate
- heating blocks or thermocyclers used for reaction incubations and/or library amplification.

Safe stopping points

The library construction process from RNA fragmentation through library amplification can be performed in 6 – 8 hours, depending on the number of samples being processed, and experience. If necessary, the protocol may be paused safely after any of the following steps:

- After the 2nd strand synthesis and marking cleanup, resuspend the washed beads in 15 µl of 1X A-Tailing Buffer, and store the sealed tube at 4 °C for up to 24 hours. Do not freeze the beads, as this can result in dramatic loss of DNA.
- After the first post-ligation cleanup, store the resuspended beads at 4 °C for up to 24 hours. Do not freeze the beads, as this can result in dramatic loss of DNA.
- After the second post-ligation cleanup, store the eluted, unamplified library DNA at 4 °C for up to 1 week, or at -20 °C for up to 1 month.

DNA solutions containing beads must not be frozen, and beads must not be 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, always store DNA in a buffered solution (10 mM Tris-HCl, pH 8.0) and minimize the number of freeze-thaw cycles.

Paramagnetic SPRI® beads and reaction cleanups

- Cleanup steps should be performed timeously to ensure that enzyme reactions do not proceed beyond optimal incubation times.
- This protocol has been validated using Agencourt® AMPure® XP reagent (Beckman Coulter, part number A63882). Solutions and conditions for RNA and DNA binding may differ if other beads are used.
- Observe all manufacturer's storage and handling recommendations for AMPure® XP reagent. Equilibrate to room temperature before use.
- Beads will settle gradually; ensure that they are fully resuspended before use.
- The incubation times provided for reaction cleanups are guidelines only, and may be modified as required, based on experience and equipment, in order to maximize library construction efficiency.
- The time required to completely capture magnetic 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.
- Ethanol (80%) for bead washes should be freshly prepared on the day of use.
- 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.
- 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 dramatic loss of DNA. Drying of beads at 37 °C is not recommended.
- Where appropriate, DNA should be eluted from beads in elution buffer (10 mM Tris-HCl, pH 8.0). Elution of DNA in PCR-grade water is not recommended, as DNA is unstable in unbuffered solutions.

Input RNA requirements

- The protocol has been validated for library construction from 10 – 400 ng of purified RNA (e.g. poly(A)-enriched RNA or rRNA-depleted RNA), in ≤10 µl of water.
- RNA is fragmented prior to 1st strand cDNA synthesis to minimize 3'→5' bias. Depending on the origin and quality of RNA, and the intended application, different fragmentation times and temperatures are recommended to obtain the required insert size distribution. To determine the quality of RNA, the sample may be analyzed using an Agilent Bioanalyzer RNA 6000 Pico kit.

- This protocol is compatible with purification of poly(A) RNA from full-length, total RNA using the Dynabeads® mRNA Purification Kit. For more details, refer to Appendix A.
- RNA dissolved in volumes >10 µl should be concentrated to 10 µl prior to use by either ethanol precipitation, SPRI® bead purification (e.g. RNAClean® XP, Beckman Coulter), or column-based methods (e.g. RNeasy® MinElute® Cleanup Kit, Qiagen).
- When concentrating RNA, an elution volume of 12 µl is recommended to ensure that 10 µl is available for use in this protocol.
- Note that some loss of material is inevitable when using any of the above methods to concentrate RNA.

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 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, reducing the quantity and expected insert size of the library.

Adapter design and concentration

- This protocol has been validated using standard, indexed Illumina® TruSeq™ "forked" adapters, but the kit is compatible with other adapters of similar design.
- Recommended adapter concentrations for library construction from 10 – 400 ng of RNA are provided in Table 1.
- Adapter concentration affects ligation efficiency, as well as adapter and adapter-dimer carry-over in post-ligation cleanups. The optimal adapter concentration for your workflow represents a compromise between cost and the above factors.
- While it is not necessary to adjust adapter concentrations to accommodate moderate sample-to-sample variations, we recommend using an adapter concentration that is appropriate for the amount of input RNA (see **Section 7: Adapter Ligation**).

Table 1. Recommended adapter concentrations

Quantity of starting material*	Adapter stock concentration	Adapter concentration in ligation reaction
10 – 50 ng	350 nM	25 nM
50 – 200 ng	700 nM	50 nM
200 – 400 ng	1400 nM	100 nM

* When following the protocol for poly(A) RNA purification outlined in Appendix A, assume 2% recovery of total RNA input.

Library amplification

- KAPA HiFi HotStart, the enzyme provided in the KAPA HiFi HotStart ReadyMix, is an antibody-based hot start formulation of KAPA HiFi DNA Polymerase, a novel B-family DNA polymerase engineered for increased processivity and high fidelity. KAPA HiFi HotStart DNA Polymerase has 5'→3' polymerase and 3'→5' exonuclease (proofreading) activities, but no 5'→3' exonuclease activity. The strong 3'→5' exonuclease activity results in superior accuracy during DNA amplification. The error rate of KAPA HiFi HotStart DNA Polymerase is 2.8×10^{-7} errors/base, equivalent to 1 error per 3.5×10^{6} nucleotides incorporated.
- In library amplification reactions (set up according to the recommended protocol), primers are typically depleted before dNTPs. When DNA synthesis can no longer take place due to substrate depletion, subsequent rounds of DNA denaturation and annealing result in the separation of complementary DNA strands, followed by imperfect annealing to non-complementary partners. This presumably results in the formation of so-called "daisy-chains" or tangled knots, comprising large assemblies of improperly annealed, partially double-stranded, heteroduplex DNA. These species migrate slower and are observed as secondary, higher molecular weight peaks during the electrophoretic analysis of amplified libraries. However, they are typically comprised of library molecules of the desired length, which are separated 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 a more accurate measurement of the amount of adapter-ligated molecules, even in the case of over-amplified libraries.
- Excessive library amplification can result in unwanted artifacts such as PCR duplicates, chimeric library inserts, amplification bias, etc. It is therefore best to minimize the number of library amplification cycles, while ensuring that sufficient material is generated for QC and sequencing.

- To minimize over-amplification and associated unwanted artifacts, the number of PCR cycles should be optimized to produce a final amplified library with a concentration range of 10 – 30 ng/µl, which is equivalent to 0.5 – 1.5 µg of DNA per 50 µl reaction.
- The number of cycles recommended in Table 2 should be used as a guide for library amplification. Cycle numbers may require adjustment depending on library amplification efficiency, RNA fragmentation profile, and the presence of adapter dimers.

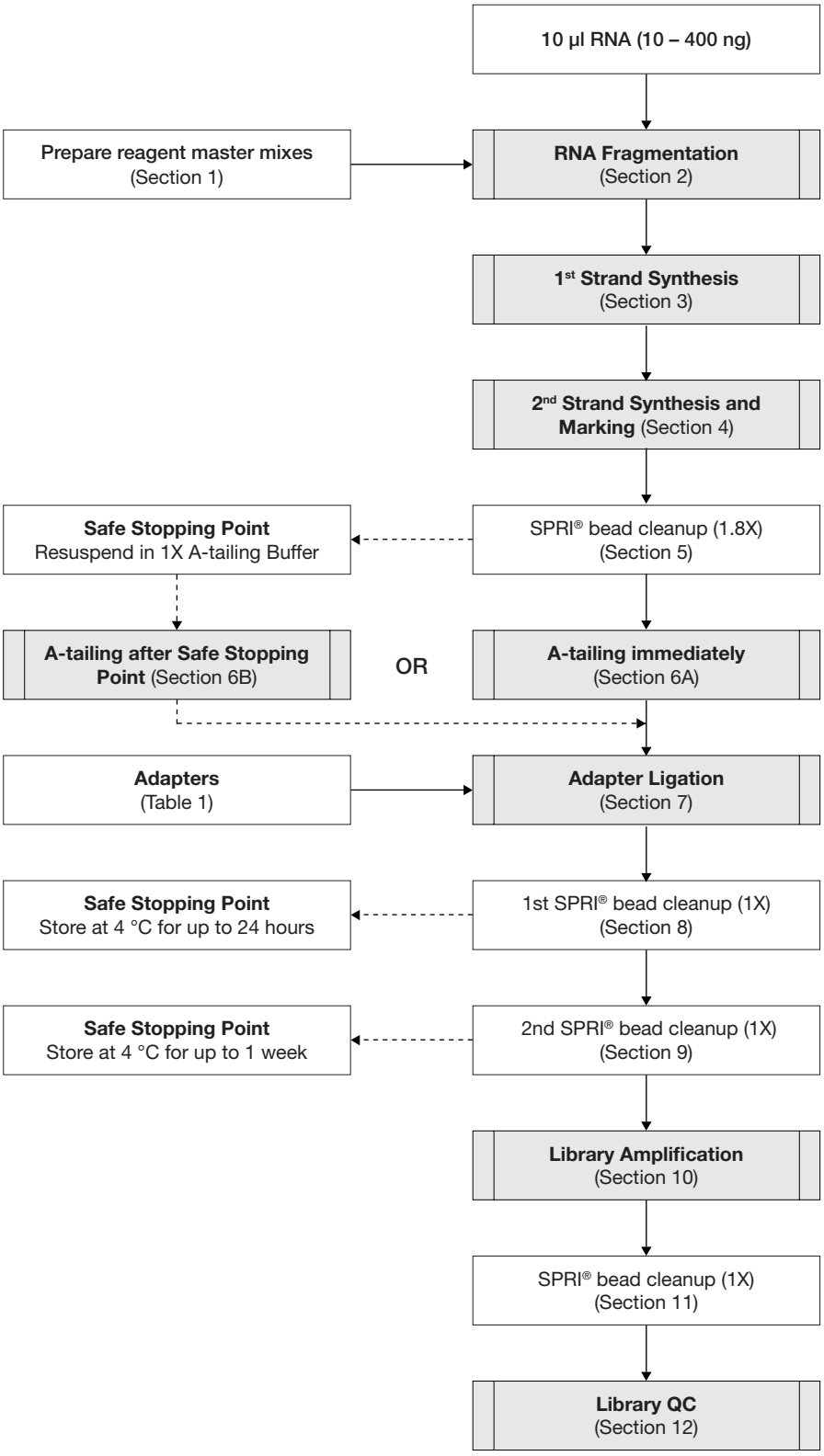
Evaluating the success of library construction

- Your specific library construction workflow should be tailored and optimized to yield a sufficient amount of adapter-ligated molecules of the desired size distribution for sequencing, QC, and archiving purposes.
- The size distribution of the double-stranded DNA and/or final amplified library should be confirmed using an electrophoretic method, whereas KAPA Library Quantification Kits for Illumina® platforms are recommended for qPCR-based quantification of libraries. These kits employ primers based on the Illumina® flow cell oligos, and can be used to quantify libraries that are ready for cluster amplification.
- Once a library construction workflow has been optimized, and consistently yields the desired amount of amplified library, it is typically not necessary to perform in-process quality control. However, qPCR-based quantification of libraries after adapter ligation or prior to library amplification can provide useful data for optimization or troubleshooting.
- Quantification of library DNA by qPCR before and after library amplification can be used to evaluate the efficiency of:
  - the process up to ligation, by determining the percentage of input RNA converted to adapter-ligated molecules
  - library amplification with the selected number of cycles, based on the actual amount of template DNA used in the PCR.
- The availability of quantification data before and after library amplification allows the two major phases of the library construction process to be evaluated and optimized independently to achieve the desired yield of amplified library.

Table 2. Recommended library amplification cycles

Input RNA	Number of cycles
10 – 50 ng	10 – 14
50 – 200 ng	8 – 12
200 – 400 ng	6 – 10

Process Workflow



Library Construction Protocol

1. Reagent preparation

This protocol takes 6 – 8 hours 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 for fragmentation and priming, 1st strand synthesis, 2nd strand synthesis and marking, A-tailing and adapter ligation are supplied separately in KAPA Stranded RNA-Seq Library Preparation Kit. For a streamlined "with-bead" protocol, a reagent master mix is prepared for each of these enzymatic steps, as outlined in Tables 3 – 7.

Volumes of additional reagents required for the KAPA Stranded RNA-Seq Library Preparation Kit protocol are listed in Table 8.

In some cases, master mixes may be constituted with varying proportions of the total final water requirement. In the examples given in the tables below, all the required water is included in each master mix, allowing the entire reaction mix to be added in a single pipetting step.

Recommendations for reaction setup at the safe stopping point after 2nd strand synthesis and marking are provided in Table 5B.

Table 3. 1st Strand Synthesis

Component	1 Library	8 Libraries	24 Libraries	96 Libraries
1st Strand Synthesis Master Mix:				
1st Strand Synthesis Buffer	11 µl	88 µl	264 µl	1 056 µl
KAPA Script	1 µl	8 µl	24 µl	96 µl
Total master mix volume:	**12 µl**	**96 µl**	**288 µl**	**1 152 µl**
Final reaction composition:				
1st Strand Synthesis Master Mix	10 µl			
Fragmented, primed RNA	20 µl			
Total reaction volume:	**30 µl**			

Table 4. 2nd Strand Synthesis and Marking

Component	1 Library	8 Libraries	24 Libraries	96 Libraries
2nd Strand Synthesis and Marking Master Mix:				
2nd Strand Marking Buffer	31 µl	248 µl	744 µl	2 976 µl
2nd Strand Synthesis Enzyme Mix	2 µl	16 µl	48 µl	192 µl
Total master mix volume:	**33 µl**	**264 µl**	**792 µl**	**3 168 µl**
Final reaction composition:				
2nd Strand Synthesis and Marking Master Mix	30 µl			
1st strand cDNA	30 µl			
Total reaction volume:	**60 µl**			

Table 5A. A-Tailing (uninterrupted protocol)

Component	1 Library	8 Libraries (10% excess)	24 Libraries (10% excess)	96 Libraries (10% excess)
A-Tailing Master Mix:				
Water	24 µl	211.2 µl	634 µl	2 534 µl
10X KAPA A-Tailing Buffer	3 µl	26.4 µl	79 µl	317 µl
KAPA A-Tailing Enzyme	3 µl	26.4 µl	79 µl	317 µl
Resuspend beads in a volume of:	**30 µl**	**264.0 µl**	**792 µl**	**3 168 µl**

Table 5B. A-Tailing (Safe Stopping Point)

Component	1 Library	8 Libraries (10% excess)	24 Libraries (10% excess)	96 Libraries (10% excess)
1X A-Tailing Buffer at Safe Stopping Point:				
Water	13.5 µl	118.8 µl	356 µl	1 426 µl
10X KAPA A-Tailing Buffer	1.5 µl	13.2 µl	40 µl	158 µl
Resuspend beads in a volume of:	**15 µl**	**132.0 µl**	**396 µl**	**1 584 µl**
A-Tailing Master Mix after Safe Stopping Point:				
Water	10.5 µl	92.4 µl	277 µl	1 109 µl
10X KAPA A-Tailing Buffer	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**	**1 584 µl**
Final reaction composition:				
Beads with dscDNA in 1X A-Tailing Buffer	15 µl			
A-Tailing Master Mix	15 µl			
Total reaction volume:	**30 µl**			

Table 6. Adapter Ligation

Component	1 Library	8 Libraries (10% excess)	24 Libraries (10% excess)	96 Libraries (10% excess)
Adapter Ligation Master Mix:				
Water	16 µl	140.8 µl	422 µl	1 690 µl
5X KAPA Ligation Buffer	14 µl	123.2 µl	370 µl	1 478 µl
KAPA T4 DNA Ligase	5 µl	44.0 µl	132 µl	528 µl
Total master mix volume:	**35 µl**	**308.0 µl**	**924 µl**	**3 696 µl**
Final reaction composition:				
Beads with A-tailed DNA	30 µl			
Adapter Ligation Master Mix	35 µl			
Adapter (350 nM – 1400 nM, as appropriate)	5 µl			
Total reaction volume:	**70 µl**			

Table 7. Library Amplification

Component	1 Library	8 Libraries (10% excess)	24 Libraries (10% excess)	96 Libraries (10% excess)
Library Amplification Master Mix:				
2X KAPA HiFi HotStart ReadyMix	25 µl	220 µl	660 µl	2 640 µl
10X KAPA Library Amplification Primer Mix	5 µl	44 µl	132 µl	528 µl
Total master mix volume	**30 µl**	**264 µl**	**792 µl**	**3 168 µl**
Final reaction composition				
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 8. Volumes of additional reagents required

Reagent	1 Library	8 Libraries	24 Libraries	96 Libraries
PEG/NaCl SPRI® Solution (provided in kit):				
1st Post-ligation cleanup	70 µl	560 µl	1.7 ml	6.8 ml
2nd Post-ligation cleanup	50 µl	400 µl	1.2 ml	4.8 ml
Total volume required:	**120 µl**	**960 µl**	**2.9 ml**	**11.6 ml**
Agencourt® AMPure® XP reagent (not supplied):				
2nd Strand synthesis and marking cleanup	108 µl	864 µl	2.6 ml	10.4 ml
Library amplification cleanup	50 µl	400 µl	1.2 ml	4.8 ml
Total volume required:	**158 µl**	**1 264 µl**	**3.8 ml**	**15.2 ml**
80% Ethanol (freshly prepared; not supplied):				
2nd Strand synthesis and marking cleanup	0.4 ml	3.2 ml	9.6 ml	38.4 ml
1st Post-ligation cleanup	0.4 ml	3.2 ml	9.6 ml	38.4 ml
2nd Post-ligation cleanup	0.4 ml	3.2 ml	9.6 ml	38.4 ml
Library amplification cleanup	0.4 ml	3.2 ml	9.6 ml	38.4 ml
Total volume required:	**1.6 ml**	**12.8 ml**	**38.4 ml**	**153.6 ml**
Elution buffer (10 mM Tris-HCl, pH 8.0; not supplied):				
Purification of poly(A) RNA from total RNA using Dynabeads® mRNA Purification Kit	50 µl	400 µl	1 200 µl	4.8 ml
1st Post-ligation cleanup	50 µl	400 µl	1 200 µl	4.8 ml
2nd Post-ligation cleanup	22.5 µl	180 µl	540 µl	2.2 ml
Library amplification cleanup	22 µl	176 µl	528 µl	2.2 ml
Total volume required:	**144.5 µl**	**1156 µl**	**3468 µl**	**14 ml**
Dynabeads® (optional; not supplied):				
Purification of poly(A) RNA from total RNA using Dynabeads® mRNA Purification Kit (Life Technologies part number 61006)	25 µl	200 µl	600 µl	2.4 ml

2. RNA Fragmentation

This protocol requires 10 – 400 ng of rRNA-depleted or poly(A)-enriched RNA, in 10 µl of RNase-free water. For poly(A) RNA purification using the Dynabeads® mRNA Purification kit, please refer to Appendix A.

To minimize 3'→5' bias, the RNA is fragmented using high temperature in the presence of magnesium. Depending on the origin and integrity of the input RNA, and the intended application, different RNA fragmentation protocols are provided to obtain the required insert size distribution. For intact RNA, extracted from fresh/frozen tissue, longer fragmentation is required at higher temperatures. For degraded or fragmented RNA (e.g. from older samples or formalin-fixed paraffin-embedded (FFPE) tissue), use a lower temperature and/or shorter times.

If the integrity of the RNA is unknown, analyze the sample prior to library construction (e.g. Agilent Bioanalyzer RNA 6000 Pico assay).

2.1 Assemble the Fragment, Prime and Elute reaction as follows:

Component	Volume
Purified RNA* (10 – 400 ng)	10 µl
2X Fragment, Prime and Elute Buffer	10 µl
Total reaction volume	**20 µl**

* For poly(A) RNA purification with the Dynabeads® mRNA Purification Kit, refer to Appendix A for a detailed RNA fragmentation protocol.

2.2 Keeping the plate/tubes on ice, mix thoroughly by gently pipetting the reaction up and down several times.

2.3 Perform fragmentation and priming using the appropriate fragmentation parameters below:

Input RNA	Desired insert size	Fragmentation and priming
Intact	100 – 200 bp	8 min @ 94 °C
	200 – 300 bp	6 min @ 94 °C
	300 – 400 bp	6 min @ 85 °C
Partially degraded	100 – 300 bp	1 – 6 min @ 85 °C
Degraded*	100 – 200 bp	30 sec @ 65 °C

* This facilitates annealing of the random primers, and will not result in any significant additional fragmentation of the RNA.

2.4 Place the plate/tube on ice and proceed immediately to **Section 3: 1st Strand Synthesis.**

3. 1st Strand Synthesis

3.1 On ice, assemble the 1st Strand Synthesis reaction as follows:

Component	Volume
Fragmented, primed RNA	20 µl
1st Strand Synthesis Master Mix (Table 3)	10 µl
Total reaction volume	**30 µl**

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

3.3 Incubate the plate/tube 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	∞

3.4 Keep the plate/tube on ice and proceed immediately to **Section 4: 2nd Strand Synthesis and Marking.**

4. 2nd Strand Synthesis and Marking

4.1 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 4)	30 µl
Total reaction volume	**60 µl**

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

4.3 Incubate the plate/tube using the following protocol:

Step	Temp.	Duration
2nd Strand Synthesis and Marking	16 °C	60 min
HOLD	4 °C	∞

4.4 Keep the plate/tube on ice and proceed immediately to **Section 5: 2nd Strand Synthesis and Marking Cleanup.**

5. 2nd Strand Synthesis and Marking Cleanup

5.1 Perform a 1.8X SPRI® cleanup by combining the following:

Component	Volume
2nd Strand Synthesis reaction	60 µl
Agencourt® AMPure® XP reagent	108 µl
Total Volume per well	**168 µl**

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

5.3 Incubate the plate/tube at room temperature for 5 – 15 min to allow the DNA to bind to the beads.

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

5.5 Carefully remove and discard 160 µl of supernatant.

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

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

5.8 Carefully remove and discard the ethanol.

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

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

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

5.12 Allow the beads to dry at room temperature, sufficiently for all the ethanol to evaporate. **Caution: over-drying the beads may result in dramatic yield loss.**

5.13 Proceed immediately to **Section 6A: A-Tailing immediately**, or follow the Safe Stopping Point instructions below.

SAFE STOPPING POINT

Resuspend the beads in 15 µl 1X A-Tailing Buffer (Table 5B), cover the reaction and store at 4 °C for up to 24 hours. Do not freeze the samples as this will damage the AMPure® XP® beads. When ready, proceed to **Section 6B: A-Tailing after Safe Stopping Point**.

6. 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 1X A-Tailing Buffer and stored at 4 °C for up to 24 hours. Depending on your chosen workflow, proceed with either **Section 6A: A-Tailing immediately** or **Section 6B: A-Tailing after Safe Stopping Point.**

6A. A-Tailing immediately

6A.1 Assemble the A-Tailing reaction as follows:

Component	Volume
Beads with dscDNA	–
A-Tailing Master Mix (Table 5A)	30 µl
Total reaction volume	**30 µl**

6A.2 Mix thoroughly by pipetting up and down several times.

6A.3 Incubate the plate/tube using the following protocol:

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

6A.4. Proceed immediately to **Section 7: Adapter Ligation**.

6B. A-Tailing after Safe Stopping Point

6B.1 To resume library preparation, combine the following reagents to perform A-Tailing:

Component	Volume
Beads with dscDNA (in 1X A-Tailing Buffer, Table 5B)	15 µl
A-Tailing Master Mix after Safe Stopping Point (Table 5B)	15 µl
Total reaction volume	**30 µl**

6B.2 Mix thoroughly by pipetting up and down several times.

6B.3 Incubate the plate/tube using the following protocol:

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

6B.4 Proceed immediately to **Section 7: Adapter Ligation.**

7. Adapter Ligation

For the purposes of determining appropriate adapter concentrations, assume that 2% of total input RNA was recovered if you followed the protocol for poly(A) RNA purification outlined in Appendix A.

7.1 Set up the adapter ligation reactions as follows:

Component	Volume
Beads with A-tailed DNA	30 µl
Adapter Ligation Master Mix (Table 6)	35 µl
Adapters*	5 µl
Total reaction volume	**70 µl**

* Variable concentration. Refer to Table 1.

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

7.3 Incubate the plate/tube at 20 °C for 15 min.

7.4 Proceed immediately to **Section 8: First Post-Ligation Cleanup.**

8. First Post-Ligation Cleanup

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

Component	Volume
Beads with adapter-ligated DNA	70 µl
PEG/NaCl SPRI® Solution	70 µl
Total volume per well/tube	**140 µl**

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

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

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

8.5 Carefully remove and discard 135 µl of supernatant.

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

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

8.8 Carefully remove and discard the ethanol.

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

8.10 Incubate the plate/tube 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 Allow the beads to dry at room temperature, sufficiently for all the ethanol to evaporate. **Caution: over-drying the beads may result in dramatic yield loss.**

8.13 Remove the plate/tube from the magnet.

8.14 Thoroughly resuspend the beads in 50 µl of 10 mM Tris-HCl (pH 8.0).

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

SAFE STOPPING POINT

The solution with resuspended beads can be stored at 4 °C for up to 24 hours. Do not freeze the beads, as this can result in dramatic loss of DNA. When ready, proceed to **Section 9: Second Post-Ligation Cleanup**.

9. Second Post-Ligation Cleanup

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

Component	Volume
Beads with purified, adapter-ligated DNA	50 µl
PEG/NaCl SPRI® Solution	50 µl
Total volume per well/tube	**100 µl**

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

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

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

9.5 Carefully remove and discard 95 µl of supernatant.

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

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

9.8 Carefully remove and discard the ethanol.

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

9.10 Incubate the plate/tube 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 Allow the beads to dry at room temperature, sufficiently for all the ethanol to evaporate. **Caution: over-drying the beads may result in dramatic yield loss.**

9.13 Remove the plate/tube from the magnet.

9.14 Thoroughly resuspend the beads in 22.5 µl of 10 mM Tris-HCl (pH 8.0). Please refer to the relevant notes under Important Parameters.

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

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

9.17 Transfer 20 µl of the clear supernatant to a new plate/tube and proceed to **Section 10: Library Amplification.**

SAFE STOPPING POINT

The solution can be stored at 4 °C for up to 1 week, or frozen at -20 °C for up to 1 month. When ready, proceed to **Section 10: Library Amplification**.

10. Library Amplification

10.1 Assemble each library amplification reaction as follows:

Component	Volume
Purified, adapter-ligated DNA	20 µl
Library Amplification Master Mix (Table 7)	30 µl
Total reaction volume	**50 µl**

10.2 Mix well by pipetting up and down several times.

10.3 Amplify the library using the following thermal cycling profile:

Step	Temp	Duration	Cycles
Initial denaturation	98 °C	45 sec	1
Denaturation	98 °C	15 sec	Refer to Table 2
Annealing*	60 °C	30 sec	
Extension	72 °C	30 sec	
Final extension	72 °C	5 min	1
Store	4 °C	∞	1

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

10.4 Place the plate/tube on ice and proceed to **Section 11: Library Amplification Cleanup.**

11. Library Amplification Cleanup

11.1 After the PCR is completed, perform a 1X SPRI® cleanup by combining the following:

Component	Volume
Amplified library DNA	50 µl
Agencourt® AMPure® XP reagent	50 µl
Total volume per well/tube	**100 µl**

11.2 Mix thoroughly by pipetting up and down several times.

11.3 Incubate the plate/tube at room temperature for 5 – 15 min to allow the DNA to bind to the beads.

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

11.5 Carefully remove and discard 95 µl of supernatant.

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

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

11.8 Carefully remove and discard the ethanol.

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

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

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

11.12 Allow the beads to dry at room temperature, sufficiently for all the ethanol to evaporate. **Caution: over-drying the beads may result in dramatic yield loss.**

11.13 Remove the plate/tube from the magnet.

11.14 Thoroughly resuspend the dried beads in 22 µl of 10 mM Tris-HCl (pH 8.0).

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

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

11.17 Transfer 20 µl of the clear supernatant to a new plate/tube and proceed to **Section 12: Library QC.**

12. Library QC

12.1 Analyze a sample of each library using an electrophoretic method to determine the library fragment size distribution, and to detect the presence of excessive adapter dimer molecules. (e.g. Bioanalyzer High Sensitivity DNA Assay)

12.2 Quantify a sample of each library using the KAPA Library Quantification Kit for Illumina® platforms.

Appendix A: Poly(A) RNA purification with the Dynabeads® mRNA Purification Kit

This appendix describes the purification of poly(A) RNA with the Dynabeads® mRNA Purification Kit, Life Technologies part number 61006.

Poly(A) RNA is obtained from total RNA by capturing twice using Dynabeads®. Note that any other RNA containing adenosine-rich regions may also be isolated. While this procedure removes a large proportion of rRNA, isolated RNA may still comprise a significant amount of rRNA.

This protocol requires 0.5 – 5 µg of pure, intact, total RNA, in ≤50 µl of RNase-free water. Degraded or fragmented total RNA will result in significant 3'-bias.

Before starting, allow Dynabeads®, Washing Buffer B, and Binding Buffer to equilibrate to room temperature and resuspend the Dynabeads® thoroughly using a vortex mixer.

A1. 1st Poly(A) Capture

A1.1 For each poly(A) selection, pipette 25 µl of the resuspended Dynabeads® into a 0.2 ml PCR tube.

A1.2 Place the tube on the magnet and incubate at room temperature until the solution is clear. Remove and discard the supernatant.

A1.3 Remove the tube from the magnet and resuspend the Dynabeads® in 50 µl of Binding Buffer.

A1.4 Place the tube on the magnet and incubate at room temperature until the solution is clear. Remove and discard the supernatant.

A1.5 Remove the tube from the magnet and resuspend the Dynabeads® in 50 µl of Binding Buffer.

A1.6 Add 50 µl of total RNA to the 50 µl Dynabeads® suspension and thoroughly mix by pipetting up and down several times.

A1.7. Incubate the tube at 65 °C for 2 minutes, followed by 5 minutes at room temperature. During the course of the room temperature incubation, gently mix the sample twice by gently pipetting up and down several times.

A1.8 Place the mixture of Dynabeads® and RNA on the magnet and incubate at room temperature until the solution is clear. Remove the supernatant.

A1.9 Remove the beads from the magnet and resuspend thoroughly in 200 µl of Washing Buffer B by pipetting up and down several times.

A1.10 Place the tube on the magnet and incubate at room temperature until the solution is clear. Remove and discard the supernatant.

A1.11 Resuspend the beads in 50 µl of 10 mM Tris-HCl (pH 8.0)

A2. Second Poly(A) Capture

A2.1 Incubate the tube at 70 °C for 2 minutes, followed by 5 minutes at room temperature. During the course of the room temperature incubation, gently mix the sample twice by gently pipetting up and down several times.

A2.2 Add 50 µl of Binding Buffer to the RNA and Dynabeads® solution and mix gently by pipetting.

A2.3 Incubate the tube at room temperature for 5 min.

A2.4 Place the tube on the magnet and incubate at room temperature until the solution is clear. Remove and discard the supernatant.

A2.5 Remove the beads from the magnet and resuspend in 200 µl of Washing Buffer B by pipetting up and down several times.

A2.6 Place the tube on the magnet and incubate at room temperature until the solution is clear. Remove and discard the supernatant.

A3. RNA Fragmentation

A3.1 Prepare the required volume of 1X Fragment, Prime and Elute Buffer as follows:

Component	Volume per sample
Water	11 µl
Fragment, Prime and Elute Buffer (2X)	11 µl
Total Volume	**22 µl**

A3.2 Thoroughly resuspend the Dynabeads® in 22 µl of 1X Fragment, Prime and Elute Buffer. Place the reaction(s) on ice, and proceed immediately.

A3.3 Place the tubes/plate in a thermal cycler and carry out the fragmentation and priming program as follows:

Desired insert size	Fragmentation and priming
100 – 200 bp	8 min @ 94 °C
200 – 300 bp	6 min @ 94 °C
300 – 400 bp	6 min @ 85 °C

A3.4 When the reaction is complete, store the fragmented RNA on ice.

A3.5 Place the plate/tube on a magnet to capture the beads, and incubate until the liquid is clear.

A3.6 Carefully remove 20 µl of the supernatant containing fragmented and primed RNA into a separate plate or 0.2 ml PCR tube.

A3.7 Proceed immediately with **Section 3: 1st Strand Synthesis.**

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