KAPABIOSYSTEMS

Technical Data Sheet

KAPA Stranded mRNA-Seq Kit

Illumina® platform

KR0960 – v3.15

This Technical Data Sheet provides product information and a detailed protocol for the KAPA Stranded mRNA-Seq Kit (Illumina® platform), product codes KK8420 and KK8421.

Contents

Kit Codes and Components

KK8420 – 24 Libraries

KAPA mRNA Capture Beads	1.2 ml
KAPA mRNA Bead Binding Buffer	3.6 ml
KAPA mRNA Bead Wash Buffer	9.6 ml
KAPA RNase-free Water	6.5 ml
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

KK8421 – 96 libraries

KAPA mRNA Capture Beads	4.8 ml
KAPA mRNA Bead Binding Buffer	14.4 ml
KAPA mRNA Bead Wash Buffer	40 ml
KAPA RNase-free Water	25 ml
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 the isolation of intact poly(A) RNA from 0.1 – 4 µg of total RNA.
- Accurate strand origin information is retained in >99% of unique mapped reads.
- To prevent foaming, avoid shaking or high speed vortexing of mRNA capture beads.
- Adapters are not supplied with this kit, but can be obtained from any reputable oligonucleotide vendor.
- This protocol requires the use of Agencourt® AMPure® XP reagent (Beckman Coulter part numbers A63880, A63881, and A63882), which is not included in the kit.
- PEG/NaCl SPRI® solution is provided for "with-bead" reaction cleanups.

Product Description

The KAPA Stranded mRNA-Seq Kit for Illumina® sequencing contains all of the buffers and enzymes required for poly(A) mRNA capture and construction of stranded mRNA-Seq libraries from 0.1 – 4 µg of intact total RNA via the following steps:

1. mRNA capture using magnetic oligo-dT beads.
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 2nd 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. 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] KAPA Stranded mRNA-Seq 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

The KAPA Stranded mRNA-Seq Kit is designed for both manual and automated NGS library construction from 0.1 – 4 µg of total, intact 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
- Targeted transcriptome

Product Specifications

Shipping and storage

KAPA Stranded mRNA-Seq Kits are supplied in two boxes. Box 1 contains capture beads and buffers, and is shipped on dry ice or ice packs, depending on the destination country. Upon receipt, **store Box 1 at 4°C**. Box 2 contains enzymes and buffers for cDNA synthesis and library preparation, and is shipped on dry ice or ice packs, depending on the destination country. The contents of Box 2 are temperature-sensitive, and appropriate care should be taken during storage. Upon receipt, **store Box 2 at -20°C** in a constant temperature freezer. The 1st Strand Synthesis Buffer and PEG/NaCl SPRI® Solution supplied in Box 2 are light-sensitive, and should be protected from light during storage. When stored under these conditions and handled correctly, the kit components will retain full activity until the expiry date indicated on the kit label.

Handling

Reagents for mRNA capture (Box 1) **must be stored at 4°C**, and not at -20°C, as long-term freezing will damage the magnetic capture beads. The KAPA mRNA Capture Beads and KAPA mRNA Bead Binding Buffer may form a precipitate when refrigerated; this is normal and does not affect product performance. Equilibrate reagents for mRNA capture to room temperature and mix thoroughly before use. KAPA mRNA Capture Beads and mRNA Bead Binding Buffer contain detergent. To prevent excessive foaming, high speed vortexing and vigorous shaking should be avoided.

Reagents for cDNA synthesis and library preparation (Box 2) **must be stored at -20°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. The 1st Strand Synthesis Buffer is 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 -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. For short-term use, the PEG/NaCl SPRI® Solution may be stored at 4°C (protected from light) for up to 2 months.

Quality control

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

Important Parameters

Input RNA requirements

- This protocol has been validated for library construction from 0.1 – 4 µg total, intact RNA, in ≤50 µl of water.
- The quantity and quality of mRNA in a total RNA preparation can vary significantly between samples. An input of 0.1 – 4 µg of total RNA is recommended to ensure that sufficient mRNA is available for downstream library preparation.
- To minimize 3'→5' bias, ensure that RNA is intact, and of high quality. The use of fragmented RNA will result in strong bias towards the 3'-end of the mRNA. To determine the quality of RNA, the sample may be analyzed using an Agilent Bioanalyzer RNA 6000 Pico kit.
- RNA resuspended in volumes >50 µl should be concentrated to 50 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). 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 RNase-free water to ensure that 50 µl is available for use in this protocol.
- For library preparation using RNA that has already undergone poly(A)-capture or ribosomal depletion by other methods, use the KAPA Stranded RNA-Seq Kit, which does not contain reagents for mRNA capture. Consult the Technical Data Sheet of the KAPA Stranded RNA-Seq Kit (KR0934) for more information.

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, reducing the quantity and insert size of the library.

Magnetic mRNA capture beads

- Beads and buffers must be stored at 4°C.
- Before use, KAPA mRNA Capture Beads must be washed and resuspended in KAPA 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.
- KAPA mRNA Capture Beads and KAPA mRNA Bead Binding Buffer contains 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
  - slow mixing on a tube/bottle roller.
- Before adding the KAPA Fragment, Prime and Elute Buffer to the beads, ensure that all of the KAPA mRNA Bead Wash Buffer has been removed. Carryover of KAPA mRNA Bead Wash Buffer may inhibit 1st strand cDNA synthesis.
- After RNA fragmentation, immediately place the heat-treated sample on the magnet, and remove the supernatant as soon as the liquid has cleared. Failure to do so may result in rebinding of poly-adenylated regions of RNA to the capture beads, resulting in a loss of transcript coverage.

Safe stopping points

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

- After mRNA capture (Steps 2.6 – 3.2), the resuspended beads (in 22 µl of Fragment, Prime and Elute Buffer) may be stored at 4°C for up to 24 hours.
- After the 2nd strand synthesis and marking cleanup (Steps 6.1 – 6.13), resuspend the washed beads in 15 µl of 1X A-Tailing Buffer, and store at 4°C for up to 24 hours.
- After the 1st post-ligation cleanup (Steps 9.1 – 9.15), store the resuspended beads at 4°C for up to 24 hours.

Important Parameters (continued)

Safe stopping points (continued)

- After the 2nd post-ligation cleanup (Steps 10.1 – 10.17), store the eluted, unamplified library DNA at 4°C for up to 1 week, or at -20°C for up to 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).

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 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 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
- heating blocks or thermocyclers used for reaction incubations and/or library amplification.

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 numbers A63880, A63881, and A63882). Solutions and conditions for DNA binding may differ if other beads are used.
- Observe all manufacturer's storage and handling recommendations for Agencourt® 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 always ensure 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 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.

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 0.1 – 4 µg of purified total 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 8: Adapter Ligation**).

Table 1. Recommended adapter concentrations

Input RNA	Adapter stock concentration	Final adapter concentration
100 – 250 ng	140 nM	10 nM
251 – 500 ng	350 nM	25 nM
501 – 2000 ng	700 nM	50 nM
2001 – 4000 ng	1400 nM	100 nM

Important Parameters (continued)

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.
- 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.
- If cycled to completion (not recommended) a single 50 µl library amplification PCR, performed as described in Section 11, can produce 8 – 10 µg of amplified library. To minimize over-amplification and associated undesired artifacts, the number of amplification cycles should be tailored to produce the optimal amount of amplified library required for downstream processes. This is typically in the range of 250 ng – 1.5 µg of final, amplified library.
- 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 and the presence of adapter-dimer.

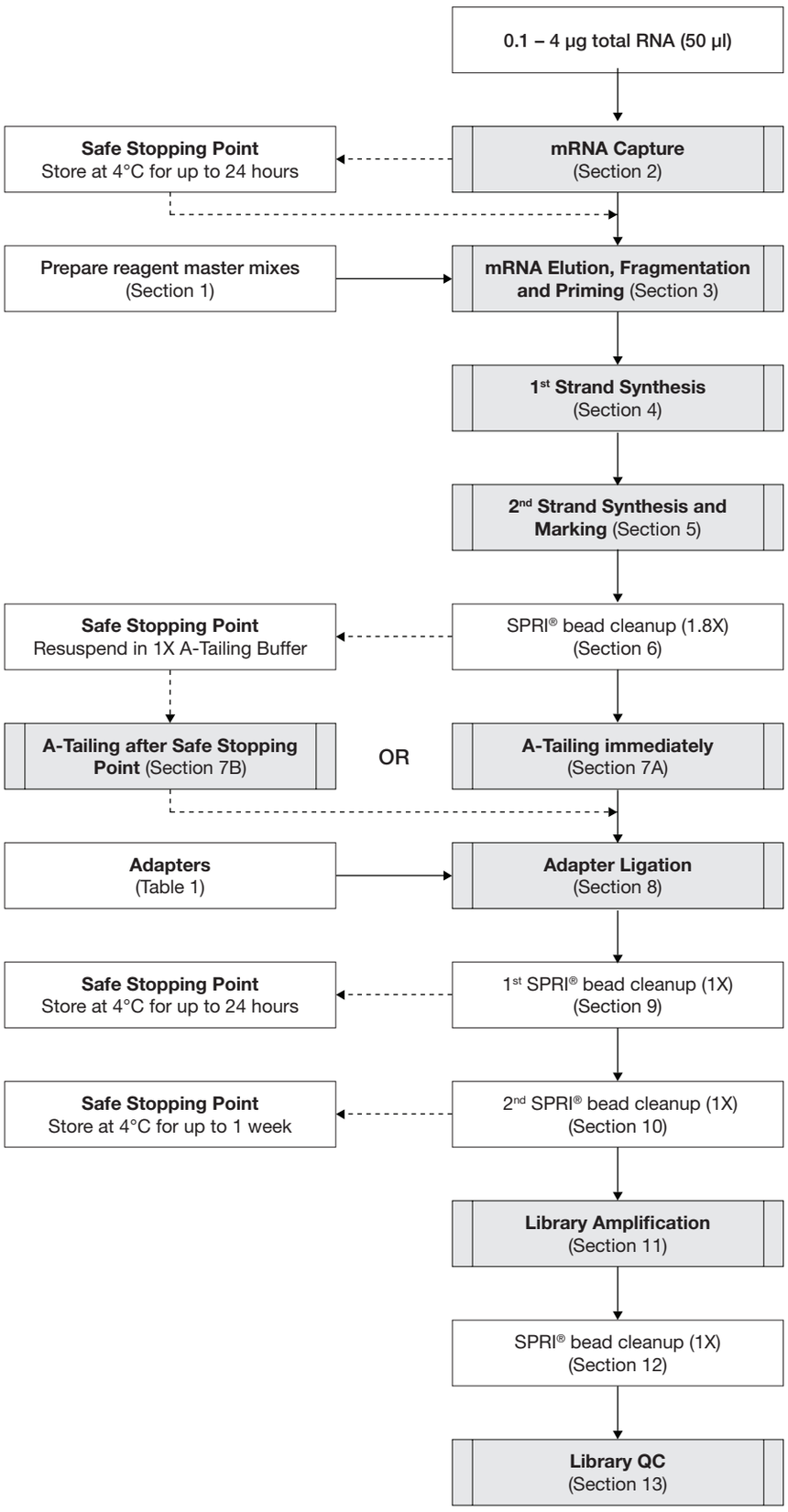
Table 2. Recommended library amplification cycles

Input RNA	Number of cycles
100 – 250 ng	10 – 16
251 – 500 ng	10 – 14
501 – 2000 ng	8 – 12
2001 – 4000 ng	6 – 10

Evaluating the success of library construction

- Your specific library construction workflow should be tailored and optimized to yield a sufficient amount of adapter-ligated molecules of the desired size distribution for sequencing, QC, and archiving purposes.
- The size distribution of 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.

Process Workflow



High quality, intact total RNA

Poly(A) RNA is captured with magnetic oligo-dT beads. Beads are resuspended in 1X Fragment, Prime and Elute Buffer

RNA is fragmented to the desired size by heating in the presence of Mg^{++}

1st strand cDNA is synthesized with random primers

2nd strand cDNA synthesis converts cDNA:RNA hybrid to dscDNA, while marking the 2nd strand with dUTP

dAMP added to 3'-end of dscDNA fragments

3'-dTMP adapters are ligated to 3'-dAMP library fragments

Adapter-ligated library DNA is amplified by PCR; dUTP-marked strand is not amplified

Library fragment size distribution is confirmed by electrophoresis; Library concentration is determined by qPCR

Library Construction Protocol

1. Reagent Preparation

This protocol takes 8 – 10 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 the KAPA Stranded mRNA-Seq 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 mRNA-Seq 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
Total master mix volume:	**30 µl**	**264.0 µl**	**792 µl**	**3 168 µl**
Resuspend beads in a volume of:	**30 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
Total master mix volume:	**15 µl**	**132.0 µl**	**396 µl**	**1 584 µl**
Resuspend beads in a volume of:	**15 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):				
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:	**94.5 µl**	**756 µl**	**2 268 µl**	**9.2 ml**

2. mRNA Capture

This protocol requires 0.1 – 4 µg of pure, intact, total RNA, in up to 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 by capturing twice using the KAPA mRNA Capture Beads. Other RNA molecules with homopolymeric adenosine regions may also be isolated.

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

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

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

2.1 Resuspend the KAPA 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: Magnetic mRNA capture beads** for more information.

2.2 For each library to be prepared, transfer 52.5 µl (50 µl + 5% excess) of the resuspended KAPA mRNA Capture Beads into an appropriate microtube. 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: Magnetic mRNA capture beads** for additional recommendations regarding bulk bead washing.

2.2 Place the microtube 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 KAPA mRNA Bead Binding Buffer (52.5 µl per library).

2.3 Remove the microtube 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.

2.4 Place the microtube 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 KAPA mRNA Bead Binding Buffer (52.5 µl per library).

2.5 Remove the microtube 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.

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

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

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

2.9 Place the plate/tube in a thermal cycler and perform the 1st mRNA capture as follows:

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

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

2.11 Remove the plate/tube from the magnet and resuspend thoroughly in 200 µl of KAPA mRNA Bead Wash Buffer by pipetting up and down several times.

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

2.13 Resuspend the beads in 50 µl of RNase-free water.

2.14 Place the plate/tube in a thermal cycler and perform the 2nd mRNA capture as follows:

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

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

2.16 Incubate the plate/tube at 20°C for 5 min.

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

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

2.19 Place the plate/tube on the magnet and incubate at room temperature until the solution is clear. Remove and discard the entire volume of supernatant.

3. mRNA Elution, Fragmentation and Priming

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

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

3.2 Thoroughly resuspend the KAPA mRNA Capture Beads with captured mRNA prepared in Step 2.13 above in 22 µl of 1X Fragment, Prime and Elute Buffer.

SAFE STOPPING POINT

Resuspended beads with captured mRNA may be stored at 4ºC for up to 24 hours. Do not freeze the samples as this will damage the beads. When ready, proceed to Step 3.3 below.

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

Desired insert size	Temp.	Duration
100 – 200 bp	94°C	8 min
200 – 300 bp	94°C	6 min
300 – 400 bp	85°C	6 min

3.4 Immediately place the plate/tube 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 Proceed immediately to **Section 4: 1st Strand Synthesis.**

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 3)	10 µl
Total volume	**30 µl**

4.2 Keeping the plate/tube on ice, 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
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 on ice and proceed immediately to **Section 5: 2nd Strand Synthesis and Marking.**

5. 2nd Strand Synthesis and Marking

5.1 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 volume	**60 µl**

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

5.3 Incubate the plate/tube using the following protocol:

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

5.4 Place the plate/tube on ice and proceed immediately to **Section 6: 2nd Strand Synthesis and Marking Cleanup.**

6. 2nd Strand Synthesis and Marking Cleanup

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

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

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

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

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

6.5 Carefully remove and discard 160 µl of supernatant.

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

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

6.8 Carefully remove and discard the ethanol.

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

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

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

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

6.13 Proceed immediately to **Section 7A: A-Tailing immediately**, or follow the Safe Stopping Point instructions on the next page.

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 7B: A-Tailing after Safe Stopping Point**.

7. A-Tailing

A-Tailing is performed either directly after the 2nd Strand Synthesis and Marking Cleanup, or after the Safe Stopping Point, where beads were resuspended in 1X A-Tailing Buffer and stored at 4°C for up to 24 hours. Depending on your chosen workflow, proceed with either **Section 7A: A-Tailing immediately** or **Section 7B: A-Tailing after Safe Stopping Point.**

7A. A-Tailing immediately

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

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

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

7A.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	∞

7A.4. Proceed immediately to **Section 8: Adapter Ligation**.

7B. A-Tailing after Safe Stopping Point

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

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

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

7B.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	∞

7B.4 Proceed immediately to **Section 8: Adapter Ligation.**

8. Adapter Ligation

8.1 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 volume	**70 µl**

* Variable concentration. Refer to Table 1.

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

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

8.4 Proceed immediately to **Section 9: 1st Post-Ligation Cleanup.**

9. 1st Post-Ligation Cleanup

9.1 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	**140 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 135 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 Dry the beads at room temperature, until all of the ethanol has evaporated. **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 50 µl of 10 mM Tris-HCl (pH 8.0).

9.15 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 10: 2nd Post-Ligation Cleanup**.

10. 2nd Post-Ligation Cleanup

10.1 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	**100 µl**

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

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

10.4 Place the plate/tube 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 on the magnet, add 200 µl of 80% ethanol.

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

10.8 Carefully remove and discard the ethanol.

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

10.10 Incubate the plate/tube 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, until all of the ethanol has evaporated. **Caution: over-drying the beads may result in dramatic yield loss.**

10.13 Remove the plate/tube from the magnet.

10.14 Thoroughly resuspend the beads in 22.5 µl of 10 mM Tris-HCl (pH 8.0).

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

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

10.17 Transfer 20 µl of the clear supernatant to a new plate/tube and proceed to **Section 11: Library Amplification.**

SAFE STOPPING POINT

The purified, adapter-ligated library DNA may 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 11: Library Amplification**.

11. Library Amplification

11.1 Assemble each library amplification reaction as follows:

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

11.2 Mix well by pipetting up and down several times.

11.3 Amplify the library using the following 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
HOLD	4°C	∞	1

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

11.4 Place the plate/tube on ice and proceed to **Section 12: Library Amplification Cleanup.**

12. Library Amplification Cleanup

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

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

12.2 Mix thoroughly by pipetting up and down several times.

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

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

12.5 Carefully remove and discard 95 µl of supernatant.

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

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

12.8 Carefully remove and discard the ethanol.

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

12.10 Incubate the plate/tube 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, until all of the ethanol has evaporated. **Caution: over-drying the beads may result in dramatic yield loss.**

12.13 Remove the plate/tube from the magnet.

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

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

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

12.17 Transfer 20 µl of the clear supernatant to a new plate/tube and proceed to **Section 13: Library QC.**

13. Library QC

13.1 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).

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

Restrictions and Liabilities

This document is provided “as is” and Kapa Biosystems assumes no responsibility for any typographical, technical, or other inaccuracies in this document. Kapa Biosystems reserves the right to periodically change information that is contained in this document; however, Kapa Biosystems makes no commitment to provide any such changes, updates, enhancements, or other additions to this document to you in a timely manner or at all.

OTHER THAN THE LIMITED WARRANTY CONTAINED IN THIS USER GUIDE, KAPA BIOSYSTEMS MAKES NO REPRESENTATIONS, WARRANTIES, CONDITIONS OR COVENANTS, EITHER EXPRESS OR IMPLIED (INCLUDING WITHOUT LIMITATION, ANY EXPRESS OR IMPLIED WARRANTIES OR CONDITIONS OF FITNESS FOR A PARTICULAR PURPOSE, NON-INFRINGEMENT, MERCHANTABILITY, DURABILITY, TITLE, OR RELATED TO THE PERFORMANCE OR NON-PERFORMANCE OF ANY PRODUCT REFERENCED HEREIN OR PERFORMANCE OF ANY SERVICES REFERENCED HEREIN).

This document might contain references to third party sources of information, hardware or software, products, or services and/or third party web sites (collectively the “Third-Party Information”). Kapa Biosystems does not control, and is not responsible for, any Third-Party Information, including, without limitation the content, accuracy, copyright compliance, compatibility, performance, trustworthiness, legality, decency, links, or any other aspect of Third-Party Information. The inclusion of Third-Party Information in this document does not imply endorsement by Kapa Biosystems of the Third-Party Information or the third party in any way.

Kapa Biosystems does not in any way guarantee or represent that you will obtain satisfactory results from using Kapa Biosystems products as described herein. The only warranties provided to you are included in the Limited Warranty enclosed with this guide. You assume all risk in connection with your use of Kapa Biosystems products.

Kapa Biosystems is not responsible nor will be liable in any way for your use of any software or equipment that is not supplied Kapa Biosystems in connection with your use of Kapa Biosystems products.

Note to Purchaser: Limited License

KAPA Stranded mRNA-Seq Kits are developed, designed and sold exclusively for research purposes and in vitro use. Neither the product, nor any individual component, has been tested for use in diagnostics or for drug development, nor is it suitable for administration to humans or animals. Please refer to the MSDS, which is available on request.

Certain applications of this product are covered by patents issued to parties other than Kapa Biosystems and applicable in certain countries. Purchase of this product does not include a license to perform any such applications. Users of this product may therefore be required to obtain a patent license depending upon the particular application and country in which the product is used.

Licensed under U.S. Patent nos. 5,338,671 and 5,587,287 and corresponding patents in other countries.

For support please contact: **support@kapabiosystems.com**

KAPABIOSYSTEMS

Boston, Massachusetts, United States
Int. Tel: +1 781 497 2933 Tel: +1 855 KAPA BIO
Fax: +1 781 497 2934
Email: sales@kapabiosystems.com

Cape Town, South Africa
Tel: +27 21 448 8200 Fax: +27 21 448 6503
Email: sales@kapabiosystems.com