KAPABIOSYSTEMS

Technical Data Sheet

KAPA Stranded RNA-Seq Kit with RiboErase (HMR) Globin

Illumina® Platforms

KR1519 – v2.17

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

Contents

Kapa/*Roche* Kit Codes and Components		
KK8485 *08308250702* 24 libraries = **KK8400** *07962142001* + **KK8481** *07962266001* + **KK8478** *08308276001*	Hybridization Buffer	110 µL
	Hybridization Oligos (HMR)	110 µL
	Globin Hybridization Oligos (HMR)	24 µL
	Depletion Buffer	80 µL
	RNase H	55 µL
	DNase Buffer	60 µL
	DNase	55 µL
	Fragment, Prime and Elute Buffer (2X)	264 µL
	1st Strand Synthesis Buffer	264 µL
	KAPA Script	25 µL
	2nd Strand Marking Buffer	750 µL
	2nd Strand Synthesis Enzyme Mix	50 µL
	A-Tailing Buffer (10X)	80 µL
	A-Tailing Enzyme	80 µL
	Ligation Buffer (5X)	380 µL
	DNA Ligase	135 µL
	PEG/NaCl Solution	5 mL
	Library Amplification Primer Mix (10X)	138 µL
	KAPA HiFi HotStart ReadyMix (2X)	690 µL
KK8486 *08308268702* 96 libraries = **KK8401** *07962169001* + **KK8482** *07962274001* + **KK8479** *08308284001*	Hybridization Buffer	480 µL
	Hybridization Oligos (HMR)	480 µL
	Globin Hybridization Oligos (HMR)	96 µL
	Depletion Buffer	360 µL
	RNase H	240 µL
	DNase Buffer	264 µL
	DNase	240 µL
	Fragment, Prime and Elute Buffer (2X)	1.32 mL
	1st Strand Synthesis Buffer	1.32 mL
	KAPA Script	120 µL
	2nd Strand Marking Buffer	3.72 mL
	2nd Strand Synthesis Enzyme Mix	240 µL
	A-Tailing Buffer (10X)	650 µL
	A-Tailing Enzyme	360 µL
	Ligation Buffer (5X)	1.7 mL
	DNA Ligase	600 µL
	PEG/NaCl Solution	30 mL
	Library Amplification Primer Mix (10X)	600 µL
	KAPA HiFi HotStart ReadyMix (2X)	3 mL

Quick Notes

- This protocol is suitable for the depletion of ribosomal RNA (rRNA) and globin mRNA transcripts from 100 ng – 1 µg of purified, total RNA extracted from human, mouse, or rat (HMR) blood-derived samples.
- Strand origin information maintained using dUTP incorporation during second strand synthesis.
- This kit contains all the reagents needed for library construction, and high efficiency and low bias library amplification except for adapters and beads. KAPA Pure Beads and KAPA Adapters are sold separately.
- PEG/NaCl Solution is provided for “with bead” reaction cleanups.

Product Description

The KAPA Stranded RNA-Seq Kit with RiboErase (HMR) Globin for Illumina sequencing contains all of the buffers and enzymes required for depletion of rRNA and globin mRNA transcripts and the construction of stranded RNA-Seq libraries from 100 ng – 1 µg of purified, blood-derived total RNA via the following steps:

1. depletion of rRNA and globin mRNA by hybridization of complementary DNA oligonucleotides and RNase H digestion to remove RNA duplexed to DNA, followed by DNase treatment to remove DNA oligonucleotides;
2. random fragmentation using heat and magnesium;
3. 1st strand cDNA synthesis using random priming;
4. 2nd strand synthesis and marking, which converts the cDNA:RNA hybrid to double-stranded cDNA (dscDNA), and incorporates dUTP into the second cDNA strand;
5. A-tailing, to add dAMP to the 3'-ends of the dscDNA library fragments;
6. adapter ligation, where dsDNA adapters with 3'-dTMP overhangs are ligated to A-tailed library insert fragments; and
7. library amplification, to amplify library fragments carrying appropriate adapter sequences at both ends using high-fidelity, low-bias PCR. The strand marked with dUTP is not amplified, allowing strand-specific sequencing.

The kit provides all of the enzymes and buffers required for rRNA and globin mRNA depletion, cDNA synthesis, library construction and amplification. Reaction buffers are supplied in convenient formats comprising all of the required reaction components. This minimizes the risk of RNase contamination, ensures consistent and homogenous reaction composition, and improves uniformity among replicate samples. Similarly, a single enzyme mixture is provided for each step of the library construction process, reducing the number of pipetting steps.

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

1. Oyola, S.O., et al., *BMC Genomics* **13**, 1 (2012).
2. Quail, M.A., et al., *Nature Methods* **9**, 10 – 11 (2012).
3. Quail, M.A., et al., *BMC Genomics* **13**, 341 (2012).
4. Ross, M.G., et al., *Genome Biology* **14**, R51 (2013).

Product Applications

The KAPA Stranded RNA-Seq Kit with RiboErase (HMR) Globin is designed for both manual and automated NGS library construction from 100 ng – 1 µg of blood-derived total RNA.

The kit depletes both cytoplasmic (5S, 5.8S, 18S, and 28S), and mitochondrial (12S and 16S) rRNA species as well as globin mRNA transcripts. The protocol is applicable to a wide range of RNA-Seq applications, including:

- gene expression analysis of high- and low-quality RNA samples;
- single nucleotide variation (SNV) discovery;
- splice junction and gene fusion identification; and
- characterization of both polyadenyated and non-polyadenylated RNAs, including noncoding and immature RNAs.

Product Specifications

Shipping and Storage

KAPA Stranded RNA-Seq Kits with RiboErase (HMR) Globin are supplied in multiple boxes:

Contents	Storage upon receipt
rRNA depletion reagents	-15°C to -25°C
Globin Hybridization Oligos	-15°C to -25°C
cDNA synthesis and library preparation reagents	-15°C to -25°C

Boxes containing enzymes, buffers, and hybridization oligos for rRNA and globin mRNA depletion, 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 rRNA depletion reagents, Globin Hybridization Oligos, cDNA synthesis and library preparation reagents at -15°C to -25°C*** in a constant-temperature freezer. The 1st Strand Synthesis Buffer and PEG/NaCl Solution are light-sensitive and should be protected from light during storage. When stored under these conditions and handled correctly, the kit components will retain full activity until the expiry date indicated on the kit label.

Handling

The rRNA depletion reagents, Globin Hybridization Oligos, cDNA synthesis and library preparation reagents must be stored at -15°C to -25°C, as these components are temperature-sensitive. Ensure that all components have been fully thawed and thoroughly mixed before use. Keep all reaction components and master mixes on ice whenever possible during handling and preparation, unless specified otherwise.

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

KAPA HiFi HotStart ReadyMix (2X) may not freeze completely, even when stored at -15°C to -25°C. Nevertheless, always ensure that the KAPA HiFi HotStart ReadyMix is fully thawed and thoroughly mixed before use.

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

- This protocol has been validated for library construction from 100 ng – 1 µg blood-derived, purified total RNA, in 10 µL of RNase-free water.
- The quantity of rRNA and globin mRNA transcript in a total RNA sample can vary significantly between samples. An input of 100 ng – 1 µg of total RNA is recommended to ensure that sufficient rRNA/globin mRNA-depleted RNA is available for downstream library preparation.
- Total RNA in volumes >10 µL should be concentrated to 10 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 (up to 10%) is inevitable when using any of the above methods to concentrate RNA.
- When concentrating RNA, elute in 12 µL of RNase-free water to ensure that 10 µL is available for use with this protocol.
- It is recommended to assess the quality and size distribution of the input RNA prior to rRNA/globin mRNA depletion by an electrophoretic method (e.g., Agilent Bioanalyzer RNA assay).
- In some cases, inhibitors may be present in a total RNA sample that could limit the efficiency of the rRNA and globin mRNA depletion. If the presence of inhibitors is unknown or suspected, an optional, up-front 3X bead purification may be used (e.g., KAPA Pure Beads or RNAClean XP, Beckman Coulter). Depletion efficiency may also be assessed prior to library preparation and sequencing, as outlined in the **Appendix** and ***will require processing a process control (no RNase H) at the same time***.
  - Depending on sample volume, perform a 3X bead-based cleanup by combining the following:
    - Y µL RNA sample (100 ng – 1 µg with 10% excess) in RNase-free water and 3 x Y µL paramagnetic beads.
    - ***Note that some loss of material (up to 10%) is inevitable when using this method to concentrate RNA.***
    - Elute purified RNA in RNase-free water for subsequent processing.

RNA Handling

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

RNA Fragmentation

- RNA is randomly fragmented by incubating at a high temperature in the presence of magnesium before carrying out 1st strand cDNA synthesis.
- Fragmentation conditions given in the **Library Construction Protocol** should be used as a guideline. Fragmentation times may require adjustment based upon the quality and size distribution of the input RNA. It is recommended that a non-precious, representative sample of RNA be evaluated for the optimal fragmentation conditions.

Safe Stopping Points

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

- After elution in Fragment, Prime and Elute Buffer (1X), **RNA Elution, Fragmentation and Priming** (step 6.5), store the rRNA/globin mRNA-depleted material at -15°C to -25°C for ≤24 hrs.
- After **2nd Strand Synthesis and Marking Cleanup** (step 9), resuspend the washed beads in 15 µL of 1X A-Tailing Buffer, and store at 2°C to 8°C for ≤24 hrs.
- After **1st Post-ligation Cleanup** (step 12), store the resuspended beads at 2°C to 8°C for ≤24 hrs.
- After **2nd Post-ligation Cleanup** (step 13), store the eluted, unamplified library at 2°C to 8°C for ≤1 week, or at -15°C to -25°C for ≤1 month.

cDNA 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 – 9.

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 your workflow, consider compatibility with:

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

Reaction Cleanups

- This protocol has been validated for use with KAPA Pure Beads or Agencourt AMPure XP (Beckman Coulter). Solutions and conditions for nucleic acid binding may differ if other beads are used.
- Cleanup steps should be performed in a timely manner to ensure that enzymatic reactions do not proceed beyond optimal incubation times.
- Observe all the storage and handling recommendations for KAPA Pure Beads or Agencourt AMPure XP. Equilibration to room temperature is essential to achieve specified size distribution and yield of libraries.
- Beads will settle gradually; ensure that they are fully resuspended before use.
- ***To ensure optimal nucleic acid recovery, it is critical that the nucleic acid and KAPA Pure Beads are thoroughly mixed*** (by extensive up-and-down pipetting) before the nucleic acid binding incubation.
- Bead incubation times are guidelines only, and may be modified/optimized according to current protocols, previous experience, specific equipment, and samples in order to maximize library construction efficiency and throughput.
- The time required for complete capture of 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.
- The volumes of 80% ethanol for the bead washes may be adjusted to accommodate smaller reaction vessels and/or limited pipetting capacity, but it is important that the beads are entirely submerged during the wash steps. ***Always use freshly prepared 80% ethanol.***
- It is important to remove all ethanol before proceeding with subsequent reactions. However, over-drying of beads may make them difficult to resuspend, and result in a dramatic loss of sample. With optimized aspiration of ethanol, drying of beads for 3 – 5 min at room temperature should be sufficient. ***Drying of beads at 37°C is not recommended.***
- Where appropriate, DNA should be eluted from beads in elution buffer (10 mM Tris-HCl, pH 8.0 – 8.5). Elution of DNA in PCR-grade water is not recommended, as DNA is unstable in unbuffered solutions. Purified DNA in elution buffer should be stable at 2°C to 8°C for 1 – 2 weeks, or at -15°C to -25°C for long-term storage. The long-term stability of library DNA at -15°C to -25°C depends on a number of factors, including library concentration. Always use low DNA-binding tubes for long-term storage, and avoid excessive freezing and thawing.

Adapter Design and Concentration

- KAPA Adapters are recommended for use with the KAPA Stranded RNA-Seq Kit with RiboErase (HMR) Globin. 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 and ordering, please contact Technical Support at **sequencing.roche.com/support**.
- Adapter concentration affects ligation efficiency, as well as adapter and adapter-dimer carry-over during post-ligation cleanups. The optimal adapter concentration for your workflow represents a compromise between the above factors and cost.
- Adapter quality has an impact on the effective concentration of adapter available for ligation. Always source the highest quality adapters from a reliable supplier, dilute and store adapters in a buffered solution with the requisite ionic strength, and avoid excessive freezing and thawing of adapter stock solutions.
- Adapter-dimer formation may occur when using highly degraded RNA inputs, or input amounts lower than the validated range (100 ng). If adapter-dimers are present, seen as a sharp 120 to 140 bp peak in the final library, these can be removed by performing a second 1X bead cleanup post amplification. 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 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. The primer mix is suitable for the amplification of all Illumina libraries flanked by the P5 and P7 flow cell sequences. Primers are supplied at a 10X concentration of 20 µM each, and have been formulated as described below. User-supplied primer mixes may be used in combination with incomplete or custom adapters. Please contact Technical Support at **sequencing.roche.com/support** for guidelines on the formulation of user-supplied library amplification primers.
- To achieve optimal amplification efficiency and avoid primer depletion, it is critical to use an optimal concentration of high quality primers. Primers should be used at a final concentration of 0.5 – 4 µM each.
- Library amplification primers should be HPLC-purified and modified to include a phosphorothioate bond at the 3'-terminal of each primer (to prevent degradation by the strong proofreading activity of KAPA HiFi HotStart). Always store and dilute primers in a buffered solution (e.g., 10 mM Tris-HCl, pH 8.0 – 8.5), and limit the number of freeze-thaw cycles. To achieve the latter, store primers at 2°C to 8°C for short-term use, or as single-use aliquots at -15°C to -25°C.
- In library amplification reactions (set up according to the recommended protocol), primers are typically depleted before dNTPs. When DNA synthesis can no longer take place due to substrate depletion, subsequent rounds of DNA denaturation and annealing result in the separation of complementary DNA strands, followed by imperfect annealing to non-complementary partners. This presumably results in the formation of so-called "daisy-chains" or "tangled knots", comprising large

assemblies of improperly annealed, partially double-stranded, heteroduplex DNA. These species migrate slower and are observed as secondary, higher molecular weight peaks during the electrophoretic analysis of amplified libraries. However, they typically comprise library molecules of the desired length, which are individualized during denaturation prior to cluster amplification or probe hybridization. Since these heteroduplexes contain significant portions of single-stranded DNA, over-amplification leads to the under-quantification of library molecules with assays employing dsDNA-binding dyes. qPCR-based library quantifications methods, such as the KAPA Library Quantification assay, quantify DNA by denaturation and amplification, thereby providing an accurate measure of the amount of adapter-ligated molecules in a library—even if the library was over-amplified.

- Excessive library amplification can result in other unwanted artifacts such as amplification bias, PCR duplicates, chimeric library inserts and nucleotide substitutions. The extent of library amplification should therefore be limited as much as possible, while ensuring that sufficient material is generated for QC and downstream processing.
- If cycled to completion (***not recommended***), one 50 µL library amplification PCR—performed as described in **Library Amplification** (step 12)—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 and the presence of adapter-dimer.

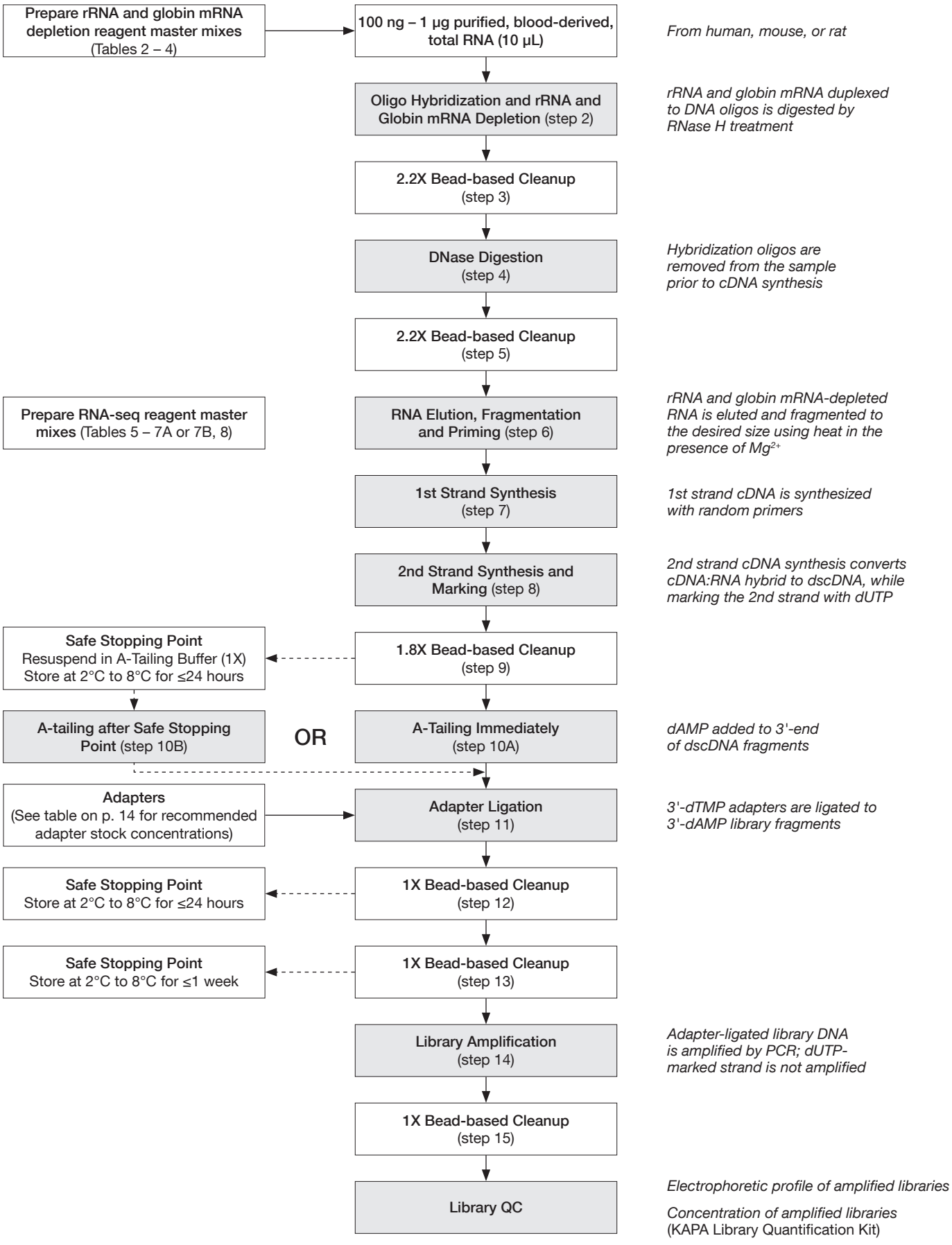
Table 1. Recommended library amplification cycles

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

Evaluating the Success of Library Construction

- The efficiency of rRNA and globin mRNA depletion may be assessed using qRT-PCR (**Appendix**).
- 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 Analytics), or similar instrument is recommended over conventional gels.
- KAPA Library Quantification Kits for Illumina platforms are recommended for qPCR-based quantification of libraries generated with the KAPA Stranded RNA-Seq Kits with RiboErase (HMR) Globin. 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 10 – 12 hrs to complete. Ideally, master mixes for the various steps in the process should be prepared as required.

For maximum stability and shelf-life, enzymes and reaction buffers are supplied separately in the KAPA Stranded RNA-Seq Kit with RiboErase (HMR) Globin. For a streamlined "with-bead" protocol, a reagent master mix with a minimum of 10% excess is prepared for each of these enzymatic steps, as outlined in Tables 2 – 9.

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

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

At the safe stopping point at A-tailing, a portion of the water and reaction buffer are added to the beads for storage at 2°C to 8°C for ≤24 hrs. To resume library construction, prepare the master mix with the remaining volume of water and reaction buffer, and the required volume of enzyme. Recommendations on how to formulate the master mix after this safe stopping point are provided in Table 7B.

Always ensure that the reagents required for oligo hybridization, rRNA/globin mRNA depletion, DNase digestion and the PEG/NaCl Solution are fully equilibrated to room temperature before use..

Table 2. Oligo hybridization

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*
Hybridization master mix:					
Hybridization Buffer	4 µL	35.2 µL	105.6 µL	422.4 µL	N*4.4 µL
Hybridization Oligos (HMR)	4 µL	35.2 µL	105.6 µL	422.4 µL	N*4.4 µL
Globin Hybridization Oligos (HMR)*	1 µL	8.8 µL	26.4 µL	105.6 µL	N*1.1 µL
RNase-free water	1 µL	8.8 µL	26.4 µL	105.6 µL	N*1.1 µL
Total master mix volume:	**10 µL**	**88 µL**	**264 µL**	**1056 µL**	**N*11 µL**
Final reaction composition:	**Per reaction**				
Hybridization master mix	10 µL				
Total RNA	10 µL				
Total reaction volume:	**20 µL**				

*If also processing non blood-derived samples, or if following the KAPA RiboErase (HMR) workflow, the volume of Globin Hybridization Oligos (HMR) must be replaced with an equal volume of RNase-free water (1 µL). For more information refer to the **KAPA Stranded RNA-Seq Kit with RiboErase (HMR)** technical data sheet – KR1151 v5.17 (or later).

Table 3. rRNA/globin mRNA depletion

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*
Depletion master mix:					
Depletion Buffer	3 µL	26.4 µL	79.2 µL	316.8 µL	N*3.3 µL
RNase H	2 µL	17.6 µL	52.8 µL	211.2 µL	N*2.2 µL
Total master mix volume:	**5 µL**	**44 µL**	**132 µL**	**528 µL**	**N*5.5 µL**
Final reaction composition:	**Per reaction**				
Depletion master mix	5 µL				
Total RNA hybridized to oligos	20 µL				
Total reaction volume:	**25 µL**				

Table 4. DNase digestion

Component:	1 library *No excess*	8 libraries *Inc. 10% excess*	24 libraries *Inc. 10% excess*	96 libraries *Inc. 10% excess*	N libraries *Inc. 10% excess*
DNase digestion master mix					
DNase Buffer	2.2 µL	19.4 µL	58 µL	232.3 µL	N*2.42 µL
DNase	2 µL	17.6 µL	52.8 µL	211.2 µL	N*2.2 µL
RNase-free water	17.8 µL	156.6 µL	469.9 µL	1879.7 µL	N*19.58 µL
Total master mix volume:	**22 µL**	**193.6 µL**	**580.8 µL**	**2323.2 µL**	**N*24.2**
Resuspend beads in a volume of:	**22 µL**				

Table 5. 1st strand synthesis

Component	1 library *Inc. 20% excess*	8 libraries *Inc. 20% excess*	24 libraries *Inc. 20% excess*	96 libraries *Inc. 20% excess*	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 enzyme	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 6. 2nd strand synthesis and marking

Component	1 library *Inc. 10% excess*	8 libraries *Inc. 10% excess*	24 libraries *Inc. 10% excess*	96 libraries *Inc. 10% excess*	N libraries *Inc. 10% excess*
2nd strand synthesis and marking 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 7A. A-tailing (uninterrupted protocol)

Component:	1 library	8 libraries *Inc. 10% excess*	24 libraries *Inc. 10% excess*	96 libraries *Inc. 10% excess*	N libraries *Inc. 10% excess*
A-tailing master mix					
Water	24 µL	211.2 µL	633.6 µL	2534.4 µL	N*26.4 µL
A-Tailing Buffer (10X)	3 µL	26.4 µL	79.2 µL	316.8 µL	N*3.3 µL
A-Tailing Enzyme	3 µL	26.4 µL	79.2 µL	316.8 µL	N*3.3 µL
Total master mix volume:	**30 µL**	**264.0 µL**	**792 µL**	**3168 µL**	N*33 µL
Resuspend beads in a volume of:	**30 µL**				

Table 7B. A-tailing (safe stopping point)

Component:	1 library	8 libraries *Inc. 10% excess*	24 libraries *Inc. 10% excess*	96 libraries *Inc. 10% excess*	N libraries *Inc. 10% excess*
A-Tailing Buffer (1X) at safe stopping point					
Water	13.5 µL	118.8 µL	356.4 µL	1425.6 µL	N*14.85 µL
A-Tailing Buffer (10X)	1.5 µL	13.2 µL	39.6 µL	158.4 µL	N*1.65 µL
Total master mix volume:	**15 µL**	**132.0 µL**	**396 µL**	**1584 µL**	**N*16.5 µL**
Resuspend beads in a volume of:	**15 µL**				
Component:	**1 library**	**8 libraries** *Inc. 10% excess*	**24 libraries** *Inc. 10% excess*	**96 libraries** *Inc. 10% excess*	**N libraries** *Inc. 10% excess*
A-tailing master mix after safe stopping point					
Water	10.5 µL	92.4 µL	277.2 µL	1108.8 µL	N*11.55 µL
A-Tailing Buffer (10X)	1.5 µL	13.2 µL	39.6 µL	158.4 µL	N*1.65 µL
A-Tailing Enzyme	3.0 µL	26.4 µL	79.2 µL	316.8 µL	N*3.3 µL
Total master mix volume:	**15 µL**	**132.0 µL**	**396 µL**	**1584 µL**	**N*16.5 µL**
Final reaction composition:	**Per reaction**				
Beads with dscDNA in A-Tailing Buffer (1X)	15 µL				
A-tailing master mix	15 µL				
Total reaction volume:	**30 µL**				

Table 8. Adapter ligation

Component:	1 library	8 libraries *Inc. 10% excess*	24 libraries *Inc. 10% excess*	96 libraries *Inc. 10% excess*	N libraries *Inc. 10% excess*
Adapter ligation master mix					
Water	16 µL	140.8 µL	422.4 µL	1689.6 µL	N*17.6 µL
Ligation Buffer (5X)	14 µL	123.2 µL	369.6 µL	1478.4 µL	N*15.4 µL
DNA Ligase	5 µL	44.0 µL	132 µL	528 µL	N*5.5 µL
Total master mix volume:	**35 µL**	**308.0 µL**	**924 µL**	**3696 µL**	N*38.5 µL
Final reaction composition:	**Per reaction**				
Beads with A-tailed DNA	30 µL				
Ligation master mix	35 µL				
Adapter (140 – 280 nM, as appropriate)	5 µL				
Total reaction volume:	**70 µL**				

Table 9. Library amplification

Component	1 library *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 10. 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 (not supplied):					
rRNA/globin mRNA depletion cleanup	55 µL	490 µL	1.5 mL	5.9 mL	N*60.5 µL
DNase digestion cleanup	44 µL	390 µL	1.2 mL	4.7 mL	N*48.4 µL
2nd strand synthesis and marking cleanup	108 µL	950 µL	2.9 mL	11.4 mL	N*120 µL
Library amplification cleanup	50 µL	440 µL	1.3 mL	5.3 mL	N*55 µL
Total volume required:	**257 µL**	**2270 µL**	**6.9 mL**	**27.3 mL**	**N*283 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):					
1st post-ligation cleanup	70 µL	620 µL	1.9 mL	7.5 mL	N*77 µL
2nd post-ligation cleanup	50 µL	440 µL	1.3 mL	5.9 mL	N*55 µL
Total volume required:	**120 µL**	**1060 µL**	**3.2 mL**	**13.4 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*
80% ethanol (freshly prepared; not supplied):					
rRNA/globin mRNA depletion cleanup	0.4 mL	3.5 mL	10.6 mL	42.2 mL	N*0.44 mL
DNase digestion cleanup	0.4 mL	3.5 mL	10.6 mL	42.2 mL	N*0.44 mL
2nd strand synthesis and marking cleanup	0.4 mL	3.5 mL	10.6 mL	42.2 mL	N*0.44 mL
1st post-ligation cleanup	0.4 mL	3.5 mL	10.6 mL	42.2 mL	N*0.44 mL
2nd post-ligation cleanup	0.4 mL	3.5 mL	10.6 mL	42.2 mL	N*0.44 mL
Library amplification cleanup	0.4 mL	3.5 mL	10.6 mL	42.2 mL	N*0.44 mL
Total volume required:	**2.4 mL**	**21.0 mL**	**63.5 mL**	**253.3 mL**	**N*2.65 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. Oligo Hybridization and rRNA/globin mRNA Depletion

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

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

*ii. If also processing non blood-derived samples, or if following the KAPA RiboErase (HMR) workflow, the volume of Globin Hybridization Oligos (HMR) must be replaced with equal volume RNase-free water. For more information, refer to the **KAPA Stranded RNA-Seq Kit with RiboErase (HMR)** technical data sheet – KR1151 v5.17 (or later).*

*iii. If rRNA and globin mRNA depletion efficiency will be assessed using qRT-PCR, **a process control (no RNase H) will be required**. Please refer to **Appendix** for more information.*

2.1 Program a thermocycler as follows:

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

2.2 Assemble rRNA/Globin mRNA Hybridization reactions as follows:

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

2.3 Mix RNA and Hybridization master mix thoroughly by pipetting up and down at least 10 times using a mixing volume of 10 µL.

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

2.5 Ensure the depletion master mix containing RNase H is added while the samples are kept at 45°C in a thermocycler. When the program reaches the pause step at 45°C, add the following to each 20 µL hybridization reaction and mix thoroughly by pipetting up and down at least 10 times using a mixing volume of 10 µL:

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

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

2.7 Proceed immediately to **rRNA/Globin mRNA Depletion Cleanup** (step 3).

3. rRNA/Globin mRNA Depletion Cleanup

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

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

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

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

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

3.5 Carefully remove and discard 75 µL of supernatant.

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

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

3.8 Carefully remove and discard the ethanol.

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

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

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

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

4. DNase Digestion

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

4.1 Assemble DNase Digestion reactions as follows:

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

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

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

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

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

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

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

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

5. DNase Digestion Cleanup

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

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

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

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

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

5.5 Carefully remove and discard 60 µL of supernatant.

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

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

5.8 Carefully remove and discard the ethanol.

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

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

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

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

6. RNA Elution, Fragmentation and Priming

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

Optional QC: *If rRNA and globin mRNA depletion efficiency is going to be assessed using qRT-PCR, a process control (no RNase H) will be required* ***and a modified RNA elution protocol*** *followed. Please refer to* ***Appendix*** *for more information.*

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

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

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

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

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

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

SAFE STOPPING POINT

Samples can be stored at -15°C to -25°C for ≤24 hrs. When ready, proceed to step 6.6.

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

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

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

7. 1st Strand Synthesis

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

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

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

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

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

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

8. 2nd Strand Synthesis and A-tailing

8.1 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 marking master mix (Table 6)	30 µL
Total volume:	**60 µL**

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

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

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

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

9. 2nd Strand Synthesis and Marking Cleanup

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

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

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

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

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

9.5 Carefully remove and discard 160 µL of supernatant.

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

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

9.8 Carefully remove and discard the ethanol.

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

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

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

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

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

SAFE STOPPING POINT

Resuspend the beads in 15 µL A-Tailing Buffer (1X) (Table 7B), cover the reaction and store at 2°C to 8°C for ≤24 hrs. **Do not freeze** the samples as this will damage the KAPA Pure Beads. When ready, proceed to **A-tailing after Safe Stopping Point** (step 10B).

10. A-tailing

A-tailing is performed either directly after **2nd Strand Synthesis and Marking Cleanup** (step 9), or after the **Safe Stopping Point**, where beads were resuspended in A-Tailing Buffer (1X) and stored at 2°C to 8°C for ≤24 hrs. Depending on your chosen workflow, proceed with either **A-tailing Immediately** (step 10A) or **A-tailing after Safe Stopping Point** (step 10B).

10A. A-tailing Immediately

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

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

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

10A.3 Incubate the plate/tube(s) using the following protocol:

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

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

10B. A-tailing after Safe Stopping Point

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

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

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

10B.3 Incubate the plate/tube(s) using the following protocol:

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

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

11. Adapter Ligation

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

Quantity of starting material	Adapter stock concentration
100 – 250 ng	140 nM
251 – 500 ng	210 nM
501 – 1000 ng	280 nM

11.2 Set up the adapter ligation reactions as follows:

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

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

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

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

12. 1st Post-ligation Cleanup

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

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

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

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

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

12.5 Carefully remove and discard 135 µL of supernatant.

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

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

12.8 Carefully remove and discard the ethanol.

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

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

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

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

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

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

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

SAFE STOPPING POINT

The solution with resuspended beads can be stored at 2°C to 8°C for ≤24 hrs. Do not freeze the beads, as this can result in dramatic loss of DNA. When ready, proceed to **2nd Post-ligation Cleanup** (step 13).

13. 2nd Post-ligation Cleanup

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

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

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

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

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

13.5 Carefully remove and discard 95 µL of supernatant.

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

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

13.8 Carefully remove and discard the ethanol.

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

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

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

13.12 Dry the beads at room temperature for 3 – 5 min, or until all of the ethanol has evaporated.

Caution: over-drying the beads may result in reduced yield.

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

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

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

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

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

SAFE STOPPING POINT

The purified, adapter-ligated library DNA may be stored at 2°C to 8°C for ≤1 week, or frozen at -15°C to -25°C for ≤1 month. When ready, proceed to **Library Amplification** (step 14).

14. Library Amplification

14.1 Assemble each library amplification reaction as follows:

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

14.2 Mix well by pipetting up and down several times.

14.3 Amplify the library using the following thermocycling profile:

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

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

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

15. Library Amplification Cleanup

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

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

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

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

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

15.5 Carefully remove and discard 95 µL of supernatant.

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

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

15.8 Carefully remove and discard the ethanol.

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

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

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

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

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

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

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

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

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

Appendix: Assessing Ribosomal RNA and Globin mRNA Depletion Efficiency using qRT-PCR

This Appendix describes how to estimate the efficiency of rRNA and globin mRNA depletion using a one-step qRT-PCR assay. The assay described in this Appendix will help to determine the relative level of depletion obtained from samples using primer sequences that target haemoglobin alpha 1 (HGA), haemoglobin beta (HGB), 28S ribosomal RNA (28S), and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). These primers can be used to determine the relative transcript abundance in blood-derived RNA samples depleted using KAPA RiboErase (HMR) Globin. Subsequently, transcript abundance is compared against a process control that is prepared without the addition of RNase H, which is responsible for degrading RNA duplexed to DNA. These assays are performed during the first Safe Stopping Point in the protocol—after elution in **1X Fragment, Prime and Elute Buffer** (step 6.5)—and allows for assessment of rRNA and globin mRNA depletion efficiency, prior to library construction. This procedure is not suitable for samples that have undergone fragmentation and priming and must be carried out directly after **1X Fragment, Prime and Elute Buffer** (step 6.5).

General recommendations

- qRT-PCR is an extremely sensitive measurement technique that is vulnerable to variation arising from a number of sources. Triplicate reactions are recommended for samples and controls, but the number of replicates may be reduced to two in order to increase throughput and reduce per-sample cost. When selecting the best strategy for a workflow and throughput requirements, keep in mind that reducing the number of replicates increases the risk of having to re-assay samples if reliable data was not obtained.
- While this protocol suggests performing a minimum of two QC assays (28S and HGA), the number of assays may be increased to include HGB and GAPDH for greater reliability.
- The primer sequences were designed to target human GAPDH, HGA, HGB, and 28S, but they may also be used to assess depletion efficiency in mouse and rat RNA samples.
- While this protocol specifies 20 µL reactions, volumes may be scaled down to 10 µL, if required.
- The process control (no RNase H) is only required for the qRT-PCR assay and does not need to be included in the downstream KAPA Stranded RNA-Seq workflow or subsequent sequencing run

Additional materials required (not supplied)

1. KAPA SYBR Fast One-Step qRT-PCR Kit (refer to Table A1 for ordering information)
2. Primer pair targeting human 28S locus (Table A2)
3. Primer pair targeting human HGA locus (Table A2)
4. Additional primer assays (optional):
   a. Primer pair targeting a human reference gene, GAPDH (Table A2)
   b. Primer pair targeting a human HGB locus (Table A2).

Table A1. Ordering information*,**

Kapa/*Roche* Kit Codes and Components			
KAPA SYBR FAST One-Step qRT-PCR Master Mix (2X) Universal qPCR Master Mix (2X) ROX High Reference Dye (50X) ROX Low Reference Dye (50X) dUTP (10 mM) KAPA RT Mix (50X)	**KK4650**	*07959613001*	100 x 20 µL reactions
	KK4651	*07959621001*	500 x 20 µL reactions
	KK4652	*07959630001*	1000 x 20 µL reactions
KAPA SYBR FAST One-Step qRT-PCR Master Mix (2X) ABI Prism™ qPCR Master Mix (2X) with ROX High incorporated dUTP (10 mM) KAPA RT Mix (50X)	**KK4660**	*07959656001*	100 x 20 µL reactions
	KK4661	*07959664001*	500 x 20 µL reactions
	KK4662	*07959672001*	1000 x 20 µL reactions
KAPA SYBR FAST One-Step qRT-PCR Master Mix (2X) LightCycler® 480 qPCR Master Mix (2X) with no passive reference dye dUTP (10 mM) KAPA RT Mix (50X)	**KK4680**	*07959753001*	100 x 20 µL reactions
	KK4681	*07959761001*	500 x 20 µL reactions
	KK4682	*07959770001*	1000 x 20 µL reactions
KAPA SYBR FAST One-Step qRT-PCR Master Mix (2X) Bio-Rad iCycler qPCR Master Mix (2X) with fluorescein incorporated dUTP (10 mM) KAPA RT Mix (50X)	**KK4670**	*07959702001*	100 x 20 µL reactions
	KK4671	*07959729001*	500 x 20 µL reactions
	KK4672	*07959737001*	1000 x 20 µL reactions

*For US and Japanese customers, please contact your local Roche Diagnostics Representative for a quote.

For customers in other regions, please contact your local Merck Representative or visit **www.sigmaaldrich.com/kapa for a quote.

Table A2. Primer sequences for rRNA and globin mRNA depletion QC assays*

qRT-PCR Assay Name	Primer Sequences (5'→3')	Product Length
GAPDH	Forward 5'– ACCATCTTCCAGGAGCGAGA– 3' Reverse 5'– ATGGTGGTGAAGACGCCAGT– 3'	92
28S	Forward 5'– TACCGGCACGAGACCGATAG– 3' Reverse 5'– TTAACGGTTTCACGCCCTCTT– 3'	91
HGA	Forward 5'– CGGTCAACTTCAAGCTCCTAAG– 3' Reverse 5'– GGCTCCAGCTTAACGGTATTTG– 3'	152
HGB	Forward 5'– CTTCAGGCTCCTGGGCAAC– 3' Reverse 5'– GACAGCAAGAAAGCGAGCTTAG– 3'	154

*Standard desalted primers are sufficient for this assay

Procedure

Process Control (No RNase H)

Provision will need to be made for a process control (no RNase H) prior to commencing the KAPA RiboErase (HMR) Globin workflow.

- For each biological sample/sample type/input amount, dilute 100 ng – 1 µg of total RNA, in 10 µL of RNase-free water to act as a process control (no RNase H) sample.
- Perform **Oligo Hybridization and rRNA/globin mRNA Depletion** (step 2).
- During rRNA/globin mRNA Depletion, omit RNase H from the reaction mix for the process control ONLY, and substitute the volume with RNase-free water.
- Continue with **rRNA/Globin mRNA Depletion Cleanup** (step 3), **DNase Digestion** (step 4), and **DNase Digestion Cleanup** (step 5).
- Proceed to RNA Elution, Fragmentation and Priming as set out in the table below.

A1. RNA Elution, Fragmentation and Priming if Performing Depletion QC

A1.1 From **RNA Elution, Fragmentation and Priming** (step 6), prepare the required volume of Fragment, Prime and Elute Buffer (1X) by combining the following at room temperature:

Component	Volume
Fragment, Prime and Elute Buffer (2X)	12 µL
RNase-free Water	12 µL
Total volume:	**24 µL**

A1.2 Thoroughly resuspend the beads with purified, DNase-treated RNA in 24 µL of Fragment, Prime and Elute Buffer (1X) by pipetting up and down multiple times.

A1.3 Incubate the plate/tube(s) at room temperature for 3 min to elute RNA off the beads.

A1.4 Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.

A1.5 Carefully transfer 2 µL of supernatant into a new plate/tube(s) labeled QC and 20 µL of supernatant into a new plate/tube(s) in order to proceed with fragmentation. The QC sample may be stored at -15°C to -25°C ≤24 hrs until the qRT-PCR assay is performed.

A1.6 Once the qRT-PCR assays has been performed, continue with **RNA Elution, Fragmentation and Priming** (step 6.6) to perform the fragmentation and priming with the remainder of the supernatant (20 µL)

A2. One-step qRT-PCR Assay

A2.1 Thaw depleted RNA samples and process control (no RNase H) on ice and centrifuge briefly to collect droplets. Dilute depleted RNA samples and process control (no RNase H) by adding 98 µL RNase-free water to each sample. To mix samples containing RNA, gently pipette the reaction mixture several times. Vortexing may fragment the RNA, resulting in loss of complexity.

A2.2 Prepare a master mix containing the appropriate volume of all reaction components common to all or a subset of reactions to be performed.

A2.3 Include a No Template Control (NTC) for each assay. The NTC will enable detection of contamination of reaction components.

A2.4 Calculate the required volume of each component based on the following table:

Component	Volume	Final Conc.
PCR-grade water	up to 20 µL	N/A
KAPA SYBR FAST qPCR Master Mix (2X)*	10 µL	1X
10 µM primer mix (forward and reverse)	0.4 µL	200 nM
50X KAPA RT Mix	0.4 µL	1X
Template DNA	5 µL	-
50X ROX High/Low* (as required – instrument specific)	0.4 µL	1X

*Kits with passive reference dye incorporated in the qPCR mix: ABI Prism kits (ROX High), ROX Low kits (ROX Low), Bio-Rad iCycler kits (fluorescein). Refer to Table B1.

A2.5 Transfer the appropriate volumes of qPCR master mix, template, and primers to each well of a PCR plate/tube(s). Depending on the number of samples being assayed, the assays may be performed on separate PCR plates, ensuring that depleted RNA samples and process control (no RNase H) for each primer set are assayed in the same plate.

A2.6 Cap or seal the reaction plate/tube(s) and centrifuge briefly.

A2.7 Perform cycling conditions as described in the following table:

Step	Temp.	Duration	Cycles
Reverse transcription	42°C	5 min	Hold
Enzyme activation	95°C	2 min	Hold
Denaturation	95°C	5 sec	35
Annealing/ extension/data acquisition	60°C	20 sec	
Dissociation	According to instrument guidelines		

Data Analysis

1. Review the background-subtracted (normalized) amplification curves and the Cq scores for replicate data points (depleted RNA samples and process controls (no RNase H)), and exclude outliers. Replicate data points should differ by ≤0.2 cycles. If the data set contains many outliers, the results are likely to be unreliable and the RT-qPCR should be repeated.
2. Calculate the average Cq values for all replicate reactions.
3. Plot the average Cq values for depleted RNA samples and process control (no RNase H) for each assay on the same bar graph for comparative purposes.
   a) Ideally, the Cq value using the GAPDH primer set will be similar between the process control (no RNase H) and depleted RNA sample. This indicates conservation of GAPDH transcript level after rRNA and globin mRNA depletion.
   b) The Cq value using the 28S primer set for the rRNA depleted sample should be higher than the process control (no RNase H). This indicates a lower concentration of 28S rRNA in the depleted sample compared to the input.
   c) Similarly, the Cq values using the HGA and HGB primer sets will be higher with the depleted sample when compared to the process control (no RNase H). This indicates a lower concentration of globin mRNA transcripts present in the depleted sample as compared to the input RNA.
4. Calculate the delta Cq by subtracting the average Cq for process control (no RNase H) from the average Cq of the corresponding depleted RNA samples (Ave Cq sample – Ave Cq control). If the efficiency of the qRT-PCR is 100%, a delta Cq of 3.3 cycles will indicate a 10-fold reduction in 28S rRNA and/or globin mRNA.
5. See the following example plot:

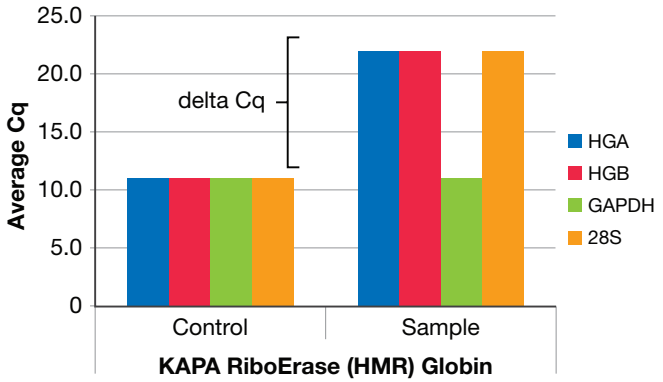


Figure 1. Comparison of average Cq values for GAPDH, 28S, HGA, and HGB assays in rRNA and globin mRNA depleted samples against a process control (no RNase H).

The KAPA SYBR Fast One-Step qRT-PCR Kit enables users to determine the relative efficiency of rRNA and globin mRNA depletion obtained from blood-derived RNA samples processed using the KAPA RiboErase (HMR) Globin Kit. While general recommendations are provided, it is critical that the minimum criteria indicating successful depletion is determined empirically. Establish and maintain a database of Cq values/delta Cq values (determined with the KAPA SYBR Fast One-Step qRT-PCR Kit or any other one-step qRT-PCR method being used), as well as residual rRNA and globin mRNA sequencing reads for different sample types and inputs. Data will enable you to define the correlation between absolute Cq and/or delta Cq values, and residual rRNA and globin mRNA species for your specific sample types, instrumentation, and workflow. This will also be invaluable for process optimization, quality control, and troubleshooting.

User may find discrepancies between residual rRNA and globin mRNA rates tabulated by qRT-PCR versus sequencing results. Reasons for such discrepancies include:

1. The theoretical average fold depletion can vary significantly for each KAPA RiboErase (HMR) Globin experiment based on starting quantity of ribosomal RNA and globin mRNA transcripts in a sample.
2. Primer sequences targeting HGA, HGB, GAPDH, and 28S were designed using different criteria than the rRNA and globin mRNA Hybridization Oligos, and so their reported qPCR values should not be interpreted as a literal estimate of the expected depletion. Rather, the purpose of this assay is to screen for potentially poor depletion results so that unproductive sequencing may be avoided.

3. Whilst the QC sample may be stored at -15°C to -25°C for ≤24 hrs until the qRT-PCR assay is performed, it is critical that dilutions are made immediately prior to setting up the assays. Calculated Cq values may be highly variable and/or inaccurate if dilute depleted RNA samples are stored at room temperature, 2°C to 8°C, or -15°C to -25°C for long periods of time prior to setting up qRT-PCRs. Furthermore, the KAPA RT Mix is temperature sensitive, and should be stored at -15°C to -25°C and kept on ice during use.
4. Low primer quality may result in loss in sensitivity due to non-specific amplification. This effect becomes more prominent at low target concentrations. The dissociation/melt curve will enable the detection of non-specific species. Always source the highest quality primer oligos from a reliable supplier, dilute and store primers in a buffered solution with the requisite ionic strength, and avoid excessive freezing and thawing of primer stock solutions.

Should a high percentage of residual rRNA and globin mRNA sequencing reads be identified, performing a 3X bead-based cleanup of the original RNA sample, as outlined in **Important Parameters: Input RNA Requirements** and repeating the RiboErase (HMR) Globin and KAPA Stranded RNA-Seq workflow is recommended.

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