INSTRUCTIONS FOR USE OF

KAPA RNA HyperPrep Kit with RiboErase (HMR)

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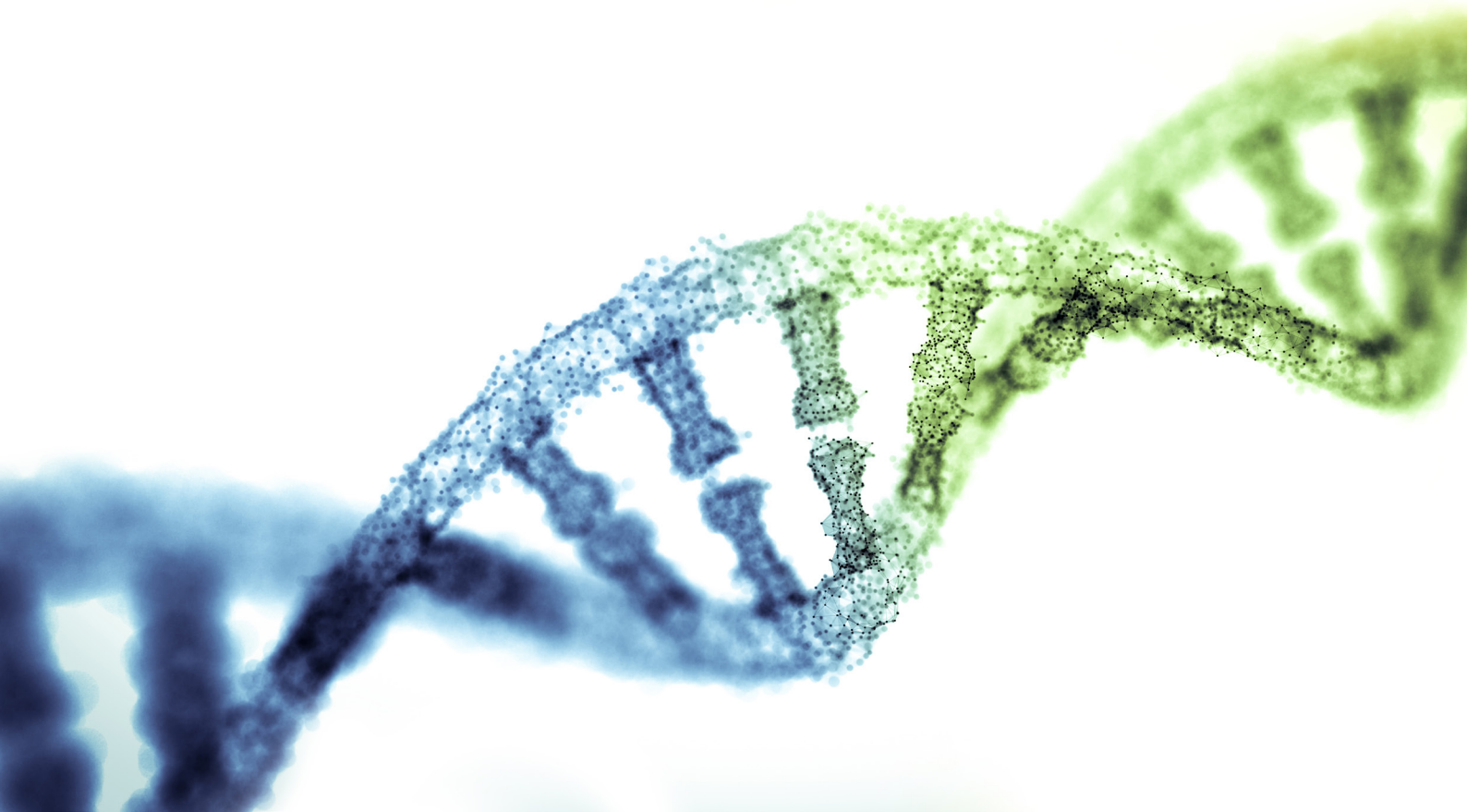
MARCH 2024, VERSION 6.0



Contents

The KAPA RNA HyperPrep Kits with RiboErase (HMR) contain:

Kit Material Number	Description	Volume
KK8560 08098131702 24 Libraries consists of the following sub kits: KK8542 08105936001 and KK8546 08105863001 and KK8482 07962266001	Hybridization Buffer	110 µL
	Hybridization Oligos (HMR)	110 µL
	Depletion Buffer	80 µL
	RNase H	55 µL
	DNase Buffer	60 µL
	DNase	55 µL
	Fragment, Prime and Elute Buffer (2X)	300 µL
	1st Strand Synthesis Buffer	300 µL
	KAPA Script	25 µL
	2nd Strand Marking Buffer	780 µL
	2nd Strand Synthesis & A-Tailing Enzyme Mix	50 µL
	Ligation Buffer	1 mL
	DNA Ligase	280 µL
	PEG/NaCl Solution	1 mL
	KAPA Pure Beads (2 x 3.2 mL bottles)	6.4 mL
	Library Amplification Primer Mix (10X)	138 µL
	KAPA HiFi HotStart ReadyMix (2X)	690 µL
KK8561 08098140702 96 Libraries consists of the following sub kits: KK8544 08105952001 and KK8001 07983280001 and KK8482 07962274001	Hybridization Buffer	480 µL
	Hybridization Oligos (HMR)	480 µL
	Depletion Buffer	360 µL
	RNase H	240 µL
	DNase Buffer	264 µL
	DNase	240 µL
	Fragment, Prime and Elute Buffer (2X)	1.4 mL
	1st Strand Synthesis Buffer	1.4 mL
	KAPA Script	130 µL
	2nd Strand Marking Buffer	3.8 mL
	2nd Strand Synthesis & A-Tailing Enzyme Mix	250 µL
	Ligation Buffer	5 mL
	DNA Ligase	1.26 mL
	PEG/NaCl Solution	5 mL
	KAPA Pure Beads	30 mL
	Library Amplification Primer Mix (10X)	600 µL
	KAPA HiFi HotStart ReadyMix (2X)	3 mL

Note: Accessory kits (KAPA Universal Adapter & KAPA UDI Primer Mixes and KAPA Unique Dual-Indexed Adapters) are sold separately.

Shipping, Storage and Stability

- KAPA Pure Beads are shipped on dry ice or ice packs, depending on the destination country. Upon receipt, store KAPA Pure Beads at +2°C to +8°C.
- Enzymes and buffers for rRNA 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 these reagents at -15°C to -25°C in a constant-temperature freezer.
- The 1st Strand Synthesis Buffer, PEG/NaCl Solution, and KAPA Pure Beads 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.

Applications

The KAPA RNA HyperPrep Kit with RiboErase (HMR or Human/Mouse/Rat) contains all of the buffers and enzymes required for depletion of ribosomal RNA (rRNA) and the rapid construction of stranded RNA-Seq libraries from 25 ng – 1 µg of purified total RNA. The KAPA RNA HyperPrep Kit with RiboErase (HMR) is designed for both manual and automated NGS library construction. The kit depletes both cytoplasmic (5S, 5.8S, 18S, and 28S), and mitochondrial (12S and 16S) rRNA species. The protocol is applicable to a wide range of RNA-Seq applications, including:

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

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

Warnings and Precautions

- Wear the appropriate personal protective equipment, such as gloves and safety glasses, to avoid direct contact while handling the reagents. Wash hands thoroughly after handling samples and reagents.
- Use good laboratory practices to avoid contamination when working with the reagents.
- Do not eat, drink, or smoke in the laboratory area.
- Do not pipette by mouth.
- In the event of a spill, clean up the solution with absorbent pads, allow pads to dry, and dispose of pads.
- Observe all national, regional, and local regulations for waste disposal and management.
- Safety Data Sheets (SDS) are available ***online*** or upon request from the local Roche office.

Ordering Information

For a complete overview of Roche Sequencing products, including KAPA RNA HyperPrep Kits with RiboErase (HMR), go to ***sequencing.roche.com*** products.

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Contact and Support

If you have questions, contact your local Roche Technical Support. Go to ***sequencing.roche.com/support*** for contact information.

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Editions

Version 1.16, October 2016; Version 2.17, September 2017; Version 2.17, October 2018 (republished); Version 3.20, December 2020; Version 4.21, April 2021; Version 5.23, June 2023; Version 6.0, March 2024

Changes to Previous Versions

Workflow modifications when using KAPA HyperPlex Adapter Kits (KAPA Universal Adapter & KAPA UDI Primer Mixes) added. Branding changes from legacy Kapa Biosystems to Roche Sequencing Solutions.

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Preface

Regulatory Disclaimer

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Contact Information

Technical Support

If you have questions, contact your local Roche Technical Support.

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Manufacturing & Distribution

Manufacturer	Roche Diagnostics Cape Town, South Africa
Distribution	Roche Diagnostics GmbH Mannheim, Germany
Distribution in USA	Roche Diagnostics Corporation Indianapolis, IN USA

Conventions Used in This Manual

Symbols

Symbols	Description
	**Important Note:** Information critical to the success of the procedure or use of the product. Failure to follow these instructions could result in compromised data.
	**Information Note:** Designates a note that provides additional information concerning the current topic or procedure.

Text

Conventions	Description
Numbered listing	Indicates steps in a procedure that must be performed in the order listed.
Italic type, blue	Highlights a resource in a different area of this manual or on a web site.
Italic type	Identifies the external general resources or names.
Bold type	Identifies names of paragraphs, sections or emphasized words.

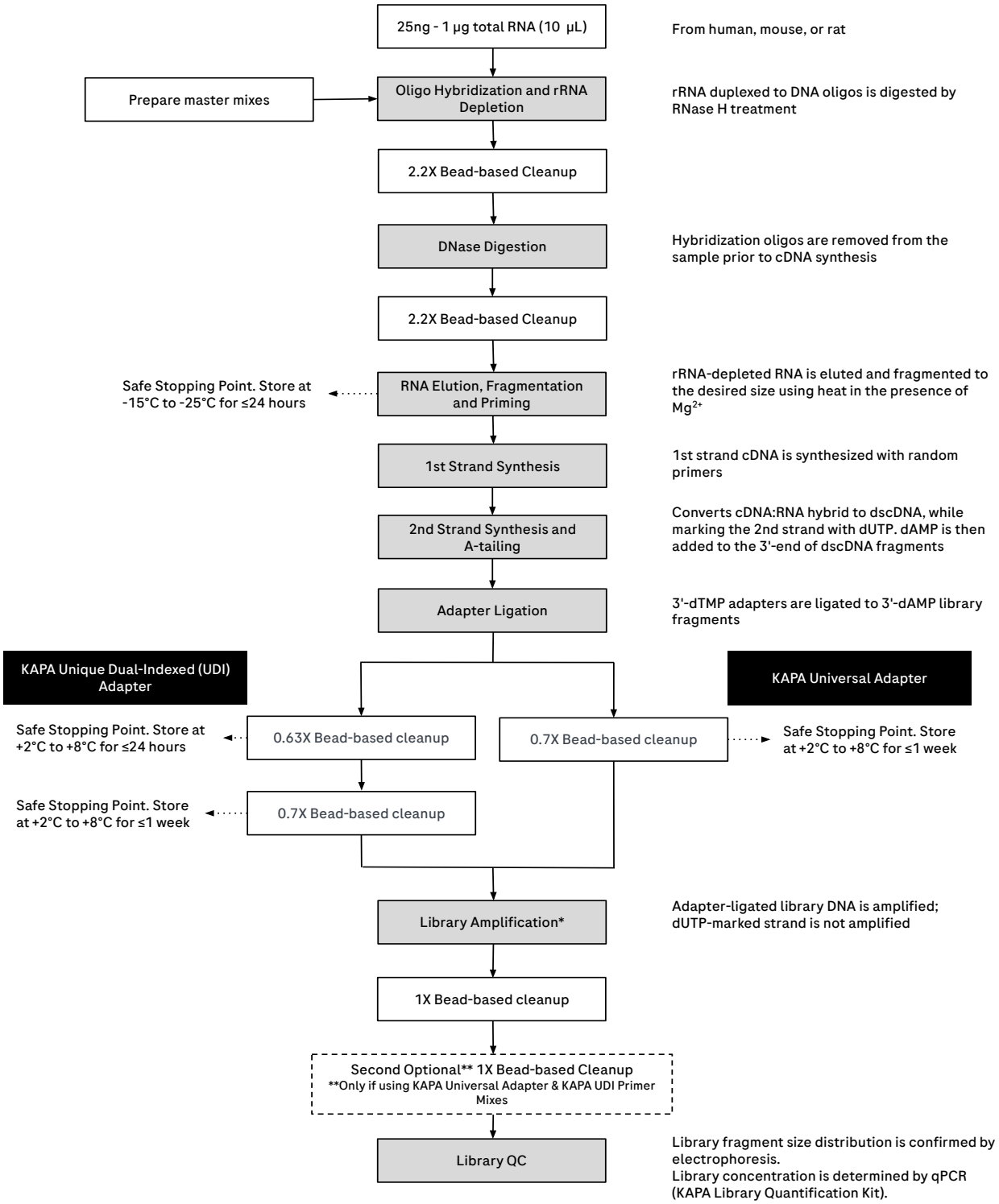
Chapter 1. Before You Begin

These Instructions for Use describe the process for depletion of ribosomal RNA (rRNA) and streamlined RNA library construction of stranded RNA libraries for the Illumina sequencing ecosystem. Specifically, these Instructions for Use provide a protocol for the workflow outlined in ***Figure 1*** using the KAPA RNA HyperPrep Kit with RiboErase (HMR).

The KAPA RNA HyperPrep Kit with RiboErase (HMR) Workflow provides:

- An easy to use, streamlined, and automation-friendly workflow with minimal resource requirements.
- Single vendor service when using the following accessory reagents:
  - KAPA Pure Beads
  - KAPA Unique Dual-Indexed (UDI) Adapters & KAPA Library Amplification Primer Mix or
  - KAPA Universal Adapter & KAPA UDI Primer Mixes 1 - 384

Figure 1 - KAPA RNA HyperPrep Kit with RiboErase (HMR) Quick Guide



*Using KAPA HiFi HS ReadyMix and **either** KAPA Library Amplification Primer Mix if KAPA UDI Adapters were used **or** KAPA UDI Primers Mixes if KAPA Universal Adapter was used.
**A modified post-amplification cleanup is highly recommended when using KAPA Universal Adapter & KAPA UDI Primer Mixes with KAPA library preparation kits. Especially if libraries will be sequenced on an Illumina NovaSeq, NextSeq 1000/2000 or HiSeq X system (with patterned flow cells). This additional or modified cleanup will serve to remove any indexed primer carryover which may exacerbate index hopping.

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.

Protocol Information & Safety

- Wear gloves and take precautions to avoid sample contamination.
- Perform all centrifugations at room temperature (+15°C to +25°C).
- Unless otherwise specified, all mixing steps are listed as 'mix thoroughly' and indicate that mixing should be performed by vortexing for at least 10 seconds.
- If liquid has collected in a tube's cap after mixing, gently tap or briefly centrifuge the sample to collect the liquid into the tube's bottom, ensuring that the mixture remains homogeneous before progressing to the next step.

References

- Thermocycler Manual
- Qubit Fluorometer Manual
- Qubit dsDNA HS Assay Kit Guide
- Qubit RNA HS Assay Kit Guide

Terminology

Sample Library: The initial shotgun library generated from RNA by fragmentation, 1st and 2nd strand synthesis, A-tailing and adapter ligation.

Amplified Sample Library: The sample library after amplification by PCR.

KAPA UDI Adapter: KAPA Unique Dual-Indexed Adapters are full-length, ready-to-use, QC-tested, dual-indexed Illumina adapters for ligation-based library construction.

KAPA UDI Primer Mixes: KAPA Unique Dual-Indexed Primer Mixes to be used in conjunction with KAPA Universal Adapters.

KAPA Universal Adapter: Truncated adapter containing a subset of sequencing motifs. Used in conjunction with KAPA UDI Primer Mixes.

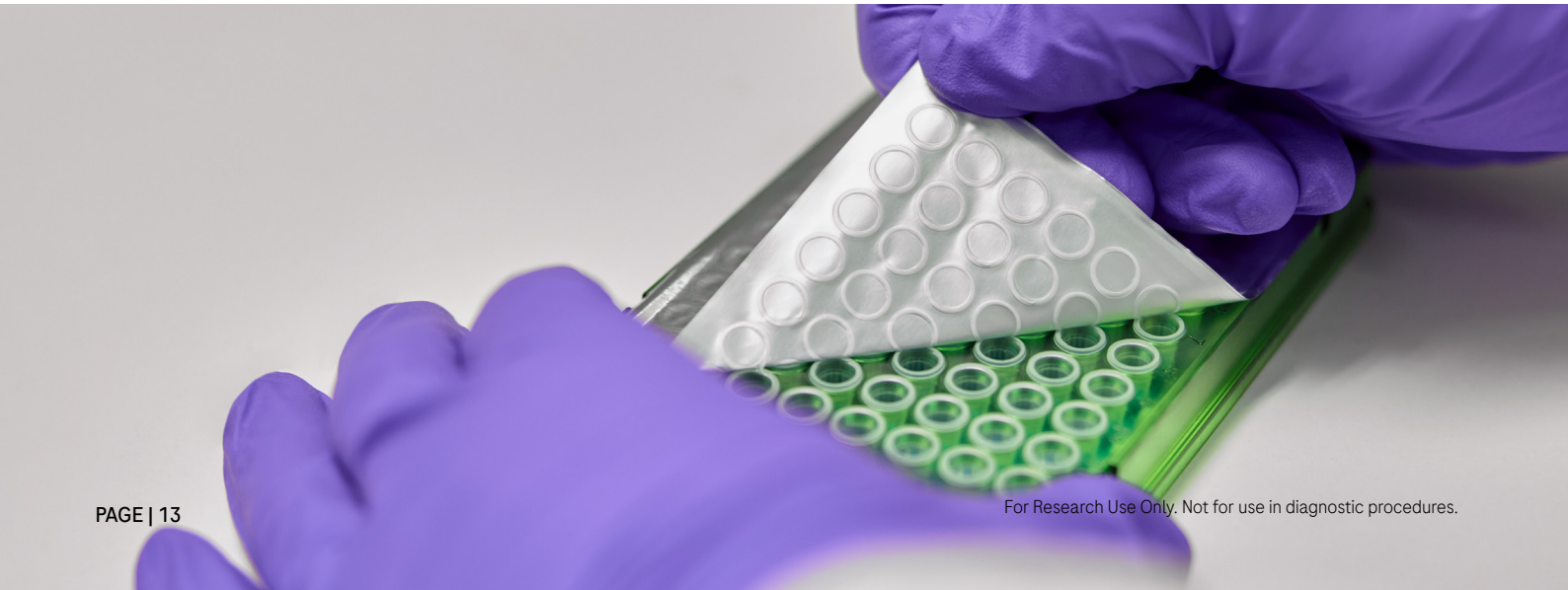
Prepare the Following Equipment and Reagents

- Thermocyclers should be programmed with the following:
  - Oligo Hybridization and rRNA Depletion program (*Chapter 3, Prepare the Sample Library, Step 1*)
  - DNase Digestion program (*Chapter 3, Prepare the Sample Library, Step 3*)
  - RNA Elution, Fragmentation and Priming program (*Chapter 3, Prepare the Sample Library, Step 5*)
  - 1st Strand Synthesis program (*Chapter 3, Prepare the Sample Library, Step 6*)
  - 2nd Strand Synthesis and A-tailing program (*Chapter 3, Prepare the Sample Library, Step 7*)
  - Adapter Ligation program (*Chapter 3, Prepare the Sample Library, Step 8*)
  - Library Amplification program (*Chapter 4, Amplify the Sample Library, Step 2*)

  It is recommended to use a thermocycler with a programmable heated lid. If not possible, please use the default settings.

- The following steps should be taken before beginning the workflow:

  Verify you are using the most up-to-date version of these Instructions for Use, go to *sequencing.roche.com/support*



Required Equipment, Labware & Consumables

You assume full responsibility when using the equipment, labware, and consumables described below. This protocol is designed for use with the specified equipment, labware, and consumables.

Laboratory Equipment

Equipment	Supplier
DynaMag-96 Side Magnet	Thermo Fisher
Microcentrifuge (16,000 x *g* capability)	Multiple Vendors
Qubit Fluorometer	ThermoFisher
Electrophoretic device & associated assays and reagents	Multiple Vendors
Thermocycler	Multiple Vendors
Vortex mixer	Multiple Vendors
Plate Centrifuge (minimum 280 x *g* capability)	Multiple Vendors

Consumables Available from Roche

Description	Package Size	Material Number
KAPA Library Quantification Kit for Illumina platforms	Various options	Various material numbers
KAPA Pure Beads	5 mL	07983271001
	30 mL	07983280001
	60 mL	07983298001
KAPA PEG/NaCl Solution	20 mL	07961928001
KAPA Unique Dual-Indexed Adapter Kit	96 x 20 µL	08861919702
KAPA Library Amplification Primer Mix	250 reactions (1.25 mL)	07958994001
KAPA Library Amplification Primer Mix	384 reactions (1.92 mL)	09420410001
KAPA Library Amplification Primer Mix 96-well plate	96 x 5 µL	09420479001
KAPA Universal Adapter	96 reactions	09063781001
	384 reactions*	09063790001
KAPA UDI Primer Mixes 1 - 96	96 reactions	09134336001
KAPA UDI Primer Mixes 97 - 192	96 reactions	09329838001
KAPA UDI Primer Mixes 193 - 288	96 reactions	09329846001
KAPA UDI Primer Mixes 289 - 384	96 reactions	09329854001

* Virtual kits - consist of 4 x 96 reaction kits

Consumables Purchased from Other Vendors

Component	Supplier
10 mM Tris-HCl, pH 8.0 – 8.5	Multiple Vendors
Ethanol, 200 proof (absolute), for molecular biology	Multiple Vendors
Qubit dsDNA HS Assay Kit & Qubit RNA HS Assay Kit	ThermoFisher
Qubit Assay Tubes	ThermoFisher
Low binding Tubes: ▪ 0.2 mL PCR tubes ▪ 1.5 mL microcentrifuge tubes (optional)	Multiple Vendors
Nuclease-free, PCR-grade water	Multiple Vendors
Agilent RNA 6000 Pico Kit	Agilent

Chapter 2.

Store and Prepare the Reagents

Step 1. Store the Reagent Kits

Reagent Kit	Storage Temperature
KAPA RNA HyperPrep Kit with RiboErase (HMR)	-15°C to -25°C
KAPA Pure Beads*	+2°C to +8°C
KAPA UDI Adapter Kit	-15°C to -25°C
KAPA Universal Adapter	-15°C to -25°C
KAPA UDI Primer Mixes or KAPA UDI Primer Mixes (resuspended)	+2°C to +8°C or -15°C to -25°C

*The KAPA Pure Beads must not be frozen.

Step 2. Prepare KAPA Adapters and KAPA UDI Primer Mixes

For multiplexing guidelines, please refer to either the *KAPA UDI Adapter* or *KAPA UDI Primer Mixes Instructions for Use* (available ***online*** on eLabDoc) as applicable.

Step 2a. Dilute the KAPA Unique Dual-Indexed Adapters (if required)

1. Retrieve the KAPA UDI Adapter plate from storage (-15°C to -25°C) and thaw at room temperature.
2. Centrifuge the KAPA UDI Adapter plate at room temperature (280 x *g* for at least 1 min) to ensure the liquid is collected at the bottom of the wells.

   Do not vortex the adapter plate as it could result in cross-contamination of the KAPA UDI Adapters. Pipette-mix individual adapters prior to use.
3. Before removing the foil cover, please ensure the plate is in the correct orientation. Please refer to the KAPA UDI Adapter Technical Data Sheet (Document number KR1736, available ***online*** on eLabDoc) for additional handling instructions.
4. Upon first use, carefully remove the foil cover of the plate to avoid cross contamination.

   Discard the original foil cover. Do not reuse.

   Maintain good lab practices when removing the foil seal. E.g., refrain from contact with the underside of the seal. If contact is made, change gloves before handling the adapter or sample plates.

   A new pipette tip must be used for each well to avoid cross contamination. If you are not using the entire contents of the KAPA UDI Adapter plate at this time, apply a new adhesive foil seal provided in the kit. Make sure that the foil is properly aligned and fully covers all 96 wells. Use a roller or other appropriate tool to ensure that the foil is evenly applied.
5. If applicable, dilute adapters to the required concentration, see table below, using the KAPA Adapter Dilution Buffer.

Step 2b. Dilute the KAPA Universal Adapters (if required)

1. Retrieve the KAPA Universal Adapters tube from storage (-15°C to -25°C) and thaw at room temperature or on ice.
2. Mix thoroughly before first use.
3. Centrifuge the KAPA Universal Adapter at room temperature (280 x *g* for at least 1 min) to ensure the liquid is collected at the bottom of the tube.
4. If applicable, dilute KAPA Universal Adapters to the required concentration, see Table 1 below, using 10 mM Tris-HCl pH 8.0 - 8.5.

Table 1. Recommended adapter concentrations for libraries constructed from different RNA sample qualities & quantities

Quantity of starting material	Quality of starting material	Adapter stock concentration *
25 - 499 ng	Partially degraded or FFPE-derived	1.5 µM
	High-quality	1.5 µM
500 - 1000 ng	Partially degraded or FFPE-derived	1.5 µM
	High-quality	7 µM

*Concentration required when using both KAPA UDI Adapter (full length) and KAPA Universal Adapter (truncated). Both KAPA UDI Adapter and KAPA Universal Adapter are provided at 15 µM.

Step 2c. Preparation of the KAPA UDI Primer Mixes

Before use of the KAPA UDI Primer Mixes, undertake the following steps to resuspend the primers:

1. Retrieve the KAPA UDI Primer Mixes plate from storage (+2°C to +8°C).
2. Centrifuge the KAPA UDI Primer Mixes plate at 280 x *g* for 1 minute to ensure the contents are at the bottom of the wells.
3. Before removing the foil cover, please ensure the plate is in the correct orientation before proceeding. In order to have well position A1 on the top left corner, the notched corner must be facing the user on the bottom left, as shown in *Figure 2*.
4. Carefully remove the foil cover on the plate ensuring to avoid cross contamination. Discard the original foil cover.
5. Using a multichannel pipette, add 10 µL of Nuclease-free, PCR-grade water directly to the bottom of each well and discard tips after dispensing.

   ⚠ A new pipette tip must be used for each well to avoid cross contamination. Be sure to dispense water slowly to the bottom of each well to avoid liquid splash over to adjacent wells.

6. Ensure every well contains 10 µL of Nuclease-free, PCR-grade water and cover the plate with one of the adhesive foil seals provided in the kit.

   ⚠ Make sure the foil seal is properly aligned and fully covers all 96 wells. Failure to do so can lead to cross contamination of the KAPA UDI Primer Mixes.

7. Use a roller or appropriate tool to ensure the foil seal is evenly applied.

8. Centrifuge the plate at 280 x *g* for 30 seconds to ensure the dispensed 10 µL are at the bottom of the well.
9. Thoroughly vortex the plate ensuring all wells are mixed well.

   ⚠ Ensure wells at the corners of the plate are mixed well by vortexing the corners of the plate. Keep the plate upright.

10. Centrifuge the plate at 280 x *g* for 1 minute to ensure the contents are collected at the bottom of the wells.
11. The KAPA UDI Primer Mixes plate is now ready for use in the ***Library Amplification*** step.
12. Store any unused but already resuspended KAPA UDI Primer Mixes at -15°C to -25°C. To avoid repeated freeze/thaw cycles you may transfer the resuspended primers to separate tubes or tube strips for storage.

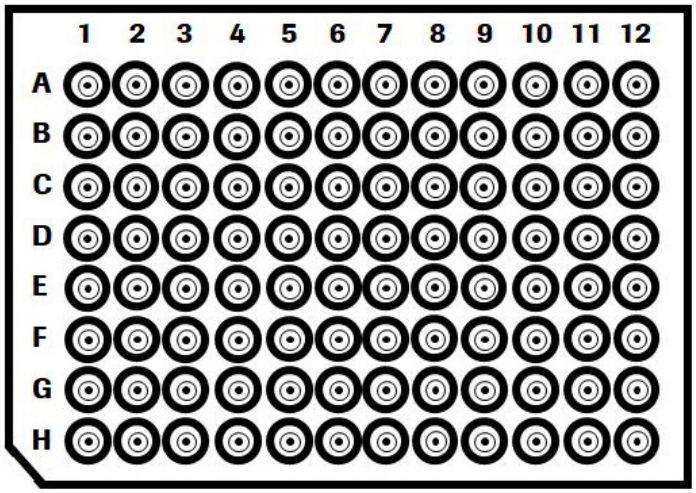


Figure 2: KAPA UDI Primer Mixes plate layout.

Considerations for Adapter Design and Concentration

- Adapter concentration affects ligation efficiency, as well as adapter and adapter-dimer carry-over during the post-ligation cleanup. The optimal adapter concentration for your workflow represents a compromise between the above factors and cost.
- When optimizing workflows for RNA inputs ≤25 ng, two or three adapter concentrations should be evaluated: try the recommended adapter concentration, as well as one or two additional concentrations in a range that is 2 – 10 times higher than the recommended concentration.
- Adapter quality has an impact on the effective concentration of adapter available for ligation. Always source the highest quality adapters from a reliable supplier, dilute and store adapters in a buffered solution with the requisite ionic strength, and avoid excessive freezing and thawing of adapter stock solutions.
- To accommodate different adapter concentrations within a batch of samples processed together, it is best to vary the concentrations of adapter stock solutions, and dispense a fixed volume (5 µL) of each adapter. The alternative (using a single stock solution, and dispensing variable volumes of adapter into ligation reactions) is not recommended.

Chapter 3.

Prepare the Sample Library

Product Description

The KAPA RNA HyperPrep Kit with RiboErase (HMR) for Illumina sequencing contains all of the buffers and enzymes required for depletion of ribosomal RNA (rRNA) and the rapid construction of stranded RNA-Seq libraries from 25 ng – 1 µg of purified total RNA via the following steps:

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

The kit provides KAPA Pure Beads for reaction cleanups, but does not include RNA or adapters. KAPA Adapters & KAPA UDI Primer Mixes are sold separately.

Reaction buffers are supplied in convenient formats comprising all of the required reaction components. This is designed to minimize the risk of RNase contamination, maximize homogeneous reaction composition and improve uniformity among replicate samples. Similarly, a single enzyme mixture is provided for each step of the library construction process, reducing the number of pipetting steps.

In order to maximize sequence coverage uniformity and to maintain relative transcript abundance, it is critical that library amplification bias be kept to a minimum. KAPA HiFi DNA Polymerase has been designed for low-bias, high-fidelity PCR, and is the polymerase of choice for NGS library amplification[1,2,3,4]. The KAPA RNA HyperPrep Kit includes 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).
4. Ross, M.G., et al., Genome Biology 14, R51 (2013).

The workflow requires the use of components from the following kits:

- KAPA RNA HyperPrep Kit with RiboErase (HMR)
- KAPA Unique Dual-Indexed Adapter Kit & KAPA Library Amplification Primers OR*
- KAPA Universal Adapter** & KAPA UDI Primer Mixes

Ensure that the following are available:

- Nuclease-free, PCR-grade water
- Freshly-prepared 80% ethanol
- 10 mM Tris-HCl, pH 8.0 - 8.5

*KAPA Unique Dual-Indexed (UDI) Adapters are recommended for use with the KAPA RNA HyperPrep Kit with RiboErase (HMR). However, the kit is also compatible with KAPA Universal Adapter & KAPA UDI Primer Mixes as well as other full-length or truncated adapter designs from 3rd party suppliers.

**The KAPA Universal UMI Adapter (Catalog# 09329862001, and provided at 33 µM), may also be considered for this workflow. The KAPA Universal UMI Adapter with Unique Molecular Identifiers (UMI) allows for molecular barcoding within each sample, enabling proper molecule counting for low-frequency variant detection.
However, the KAPA Universal UMI Adapter has only been validated within specific KAPA Target Enrichment workflows and may not confer the same computational benefits for non-target enrichment workflows (such as whole-transcriptome sequencing). Contact your local Roche Technical Support at ***sequencing.roche.com/support*** for guidance and important considerations.

Sample Requirements

- The protocol has been validated for library construction from 25 ng – 1 µg of total RNA in ≤10 µL of RNase-free water. The quantity of rRNA in a total RNA sample can vary significantly between samples. An input of 25 ng - 1 µg of total RNA is recommended to ensure that sufficient rRNA-depleted RNA is available for downstream library preparation.
- RNA in volumes >10 µL should be concentrated to 10 µL prior to use by either ethanol precipitation, bead purification (e.g., KAPA Pure Beads or RNAClean XP beads, Beckman Coulter), or column-based methods (e.g., RNeasy MinElute Cleanup Kit, QIAGEN). **Note that some loss of material is inevitable when using any of the above methods to concentrate RNA.**
- When concentrating RNA, elute in 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 depletion by an electrophoretic method (e.g., Agilent Bioanalyzer RNA assay).
- The quality of RNA extracted from formalin-fixed paraffin embedded (FFPE) tissue can be highly variable due to the damaging nature of the formalin fixation process, where crosslinking, chemical modification, and fragmentation can occur. Library construction results may vary depending on the input amount and quality of the RNA. Increasing the input amount of RNA (up to 100 ng) may salvage library construction with particularly difficult FFPE samples. For guidance on lower input amounts or sample quality, please see contact ***sequencing.roche.com/support.***

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., RNaseZAP, 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.

Important Considerations for RNA Fragmentation

- RNA is fragmented by incubating at a high temperature in the presence of magnesium before carrying out 1st strand cDNA synthesis.
- Fragmentation conditions given in the ***Chapter 3*** should be used as a guideline and may require adjustment based upon the quality and size distribution of the input RNA. It is recommended that a non-precious, representative sample of RNA be evaluated for the optimal fragmentation conditions.
- For intact RNA, such as that extracted from fresh/frozen tissue, longer fragmentation is required at higher temperatures. For degraded or fragmented RNA (e.g., from older samples or FFPE tissue), use a lower temperature and/or shorter time.
- For fragmentation optimization beyond what is provided in ***Chapter 3***, please refer to ***Appendix A: Library Size Distribution Optimization.***
- Fragmentation conditions depend on the degree of degradation. For samples with:
    - RNA Integrity Number, RIN ≥8, intact fragmentation conditions should be evaluated.
    - RIN between 3 and 7, partially degraded fragmentation conditions should be evaluated.
    - RIN less than 3, degraded fragmentation conditions should be evaluated.
- Alternatively, DV_{200} scores (proportion of molecules that are > 200 nt) can be used as an indicator of quality and is particularly helpful for FFPE samples where the RIN score might not be informative.
    - DV_{200} > 70% (high quality), intact fragmentation conditions should be evaluated.
    - DV_{200} 50 - 70% (medium quality), partially degraded fragmentation conditions should be evaluated.
    - DV_{200} 30 - 50% (low quality), degraded fragmentation conditions should be evaluated.
    - DV_{200} < 30% (too degraded), not recommended for library preparation but degraded fragmentation conditions may be evaluated.

Step 1. Oligo Hybridization and rRNA Depletion

This protocol requires 25 ng – 1 µg of total RNA, in 10 µL of RNase-free water.

⚠ Ensure that the hybridization master mix and the depletion master mix are prepared and kept at room temperature before use.

1. Pre-program a thermocycler as per the table below. Ensure that the thermocycler lid temperature is set to +105°C (default).

Step	Temperature	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. Prepare the required volume of hybridization master mix and depletion master mix as follows and keep at room temperature before use:

Oligo Hybridization Master Mix

Component	Volume Per Individual Sample
Hybridization Buffer	4 µL
Hybridization Oligos (HMR)	4 µL
RNase-free water	2 µL
Total hybridization master mix volume	**10 µL**

Depletion Master Mix

Component	Volume Per Individual Sample
Depletion Buffer	3 µL
RNase H	2 µL
Total rRNA depletion master mix volume	**5 µL**

3. Assemble the rRNA hybridization reaction as per table below, place samples in the pre-programmed thermocycler and execute the program:

Component	Volume Per Individual Sample
Total RNA in water	10 µL
Hybridization master mix (room temperature)	10 µL
Total volume	**20 µL**

4. When the program reaches the pause step at +45°C, add the Depletion master mix to each 20 µL hybridization reaction and mix thoroughly by pipetting up and down multiple times.

⚠ Ensure the depletion master mix containing RNase H is added while the samples are kept at +45°C in the thermocycler. Do NOT remove the samples from the thermocycler.

Component	Volume Per Individual Sample
Total RNA hybridized to oligos (at +45°C)	20 µL
Depletion master mix (room temperature)	5 µL
Total volume	**25 µL**

5. Resume the cycling program to continue with the depletion step (+45°C for 30 minutes).
6. Proceed immediately to *Step 2. rRNA Depletion Cleanup*

Step 2. rRNA Depletion Cleanup

1. To each rRNA depleted reaction, add 55 µL of room temperature KAPA Pure Beads that have been thoroughly resuspended as per the table below:

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

2. Once added, pipette-mix thoroughly and centrifuge briefly to collect all droplets. Do **NOT** allow beads to pellet.

⚠ It is important at this step to ensure that the solution is thoroughly mixed and appears homogeneous.

3. Incubate the sample at room temperature for 5 min to bind RNA to the beads.
4. Place the sample on a magnet to capture the beads. Incubate until the liquid is clear.
5. Carefully remove and discard 75 µL of supernatant.
6. Keeping the sample on the magnet, add 200 µL of 80% ethanol.
7. Incubate the sample on the magnet at room temperature for ≥30 sec.
8. Carefully remove and discard the ethanol.
9. Keeping the sample on the magnet, add 200 µL of 80% ethanol.
10. Incubate the sample on the magnet at room temperature for ≥30 sec.
11. Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.
12. Dry the beads at room temperature for 3 – 5 min, or until all of the ethanol has evaporated.

⚠ Over-drying the beads may result in dramatic yield loss. Over-drying is indicated by a dry, cracked appearance of the bead pellet. The bead pellet should have a matte appearance when sufficiently dried.

Step 3. DNase Digestion

To remove the hybridization oligonucleotides from the ribosomal-depleted RNA, the sample is incubated with DNase.

⚠ Ensure that the DNA digestion master mix is prepared and kept at room temperature.

⚠ Do not vortex the DNase or DNA digestion master mix. Mix thoroughly by pipetting up and down multiple times.

1. Prepare the required volume of DNase digestion master mix as follows and keep at room temperature before use:

DNase Digestion Master Mix

Component	Volume Per Individual Sample
DNase Buffer	2.2 µL
DNase	2 µL
RNase-free water	17.8 µL
Total DNase digestion master mix volume	**22 µL**

2. Assemble the DNase Digestion reactions as per table below:

Component	Volume Per Individual Sample
Beads with rRNA-depleted RNA	-
DNase digestion master mix	22 µL
Total volume	**22 µL**

3. Once added, pipette-mix thoroughly and centrifuge briefly to collect all droplets. Do NOT allow beads to pellet.

⚠ It is important at this step to ensure that the solution is thoroughly mixed and appears homogeneous.

4. Incubate the sample at room temperature for 3 min to elute the RNA off the beads.
5. Place the sample on a magnet to capture the beads. Incubate until the liquid is clear.
6. Carefully transfer 20 µL of supernatant into a new plate/tube(s). Discard the plate/tube(s) with beads.
7. Incubate the plate/tube(s) with supernatant using the following protocol. Ensure that the thermocycler lid temperature is set to +50°C.

Step	Temperature	Duration
DNase digestion	+37°C	30 min
Hold	+4°C	∞

8. Proceed immediately to *Step 4. DNase Digestion Cleanup*.

Step 4. DNase Digestion Cleanup

1. To each DNase depleted reaction, add 44 µL of room temperature KAPA Pure Beads that have been thoroughly resuspended as per the table below:

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

2. Once added, pipette-mix thoroughly and centrifuge briefly to collect all droplets. Do NOT allow beads to pellet.

   ⚠ It is important at this step to ensure that the solution is thoroughly mixed and appears homogeneous.

3. Incubate the sample at room temperature for 5 min to bind RNA to the beads.
4. Place the sample on a magnet to capture the beads. Incubate until the liquid is clear.
5. Carefully remove and discard 60 µL of supernatant.
6. Keeping the sample on the magnet, add 200 µL of 80% ethanol.
7. Incubate the sample on the magnet at room temperature for ≥30 sec.
8. Carefully remove and discard the ethanol.
9. Keeping the sample on the magnet, add 200 µL of 80% ethanol.
10. Incubate the sample on the magnet at room temperature for ≥30 sec.
11. Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.
12. Dry the beads at room temperature for 3 – 5 min, or until all of the ethanol has evaporated.

    ⚠ Over-drying the beads may result in dramatic yield loss. Over-drying is indicated by a dry, cracked appearance of the bead pellet. The bead pellet should have a matte appearance when sufficiently dried.

Step 5. RNA Elution, Fragmentation and Priming

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

1. Prepare the required volume of Fragment, Prime and Elute Buffer (1X) at room temperature as per the table below:

Component	Volume Per Individual Sample
Fragment, Prime and Elute Buffer (2X)	11 µL
RNase-free water	11 µL
Total volume	**22 µL**

2. Thoroughly resuspend the beads with purified, DNase-treated RNA in 22 µL of Fragment, Prime and Elute Buffer (1X) by gently pipetting up and down multiple times.
3. Incubate the sample at room temperature for 3 min to elute RNA off the beads.
4. Place the sample on a magnet to capture the beads. Incubate until the liquid is clear.
5. Carefully transfer 20 µL of supernatant to 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 the next step.
6. Place the plate/tube(s) in a thermocycler and carry out the fragmentation and priming program as per the table below:

   Ensure that the thermocycler lid temperature is set to +105°C (default).

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

*Refer to Important Considerations for RNA Fragmentation for guidance on RNA quality indicators.

7. Place the plate/tube(s) on ice and proceed immediately to *Step 6. 1st Strand Synthesis.*

Step 6. 1st Strand Synthesis

1. On ice, assemble the 1st strand synthesis master mix as per the table below:

1st Strand Synthesis Master Mix

Component	Volume Per Individual Sample *inc. 20% excess*
1st Strand Synthesis Buffer	11 µL
KAPA Script	1 µL
Total 1st strand synthesis master mix volume	**12 µL**

2. Keeping the plate/tube(s) on ice, combine the input RNA (fragmented & primed) and 1st strand synthesis master mix as per the table below:

Component	Volume Per Individual Sample
Fragmented, primed RNA	20 µL
1st strand synthesis master mix	10 µL
Total volume	**30 µL**

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

⚠ Ensure that the thermocycler lid temperature is set to +85°C.

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

Step	Temperature	Duration
Primer extension	+25°C	10 min
1st strand synthesis	+42°C	15 min
Enzyme inactivation	+70°C	15 min
Hold	+4°C	∞

5. Place the plate/tube(s) on ice and proceed immediately to *Step 7. 2nd Strand Synthesis & A-tailing.*

Step 7. 2nd Strand Synthesis & A-tailing

1. On ice, assemble the 2nd strand synthesis and A-tailing master mix as per table below:

2nd Strand Synthesis & A-tailing Master Mix

Component	Volume Per Individual Sample *inc. 10% excess*
2nd Strand Marking Buffer	31 µL
2nd Strand Synthesis & A-Tailing Enzyme Mix	2 µL
Total 2nd strand synthesis and A-tailing master mix volume	**33 µL**

2. Keeping the plate/tube(s) on ice, combine the 1st strand synthesis product and 2nd strand synthesis and A-tailing master mix as per the table below:

Component	Volume Per Individual Sample
1st strand synthesis product	30 µL
2nd strand synthesis & A-tailing master mix	30 µL
Total volume	**60 µL**

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

⚠ Ensure that the thermocycler lid temperature is set to +85°C.

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

Step	Temperature	Duration
2nd strand synthesis	+16°C	30 min
A-tailing	+62°C	10 min
Hold	+4°C	∞

5. Place the plate/tube(s) on ice and proceed immediately to *Step 8. Adapter Ligation*.

Step 8. Adapter Ligation

KAPA UDI Adapter and KAPA Universal Adapter, unless specified, will hereafter be referred to as KAPA Adapters.

KAPA Adapters must be added to each tube/well individually prior to addition of the Ligation reagents.

If using KAPA Universal Adapter, sample indexes are incorporated during the Library Amplification step (*Chapter 4, Amplify the Sample Library*). Precautions should be taken to avoid sample cross contamination.

1. On ice, assemble each Adapter Ligation reaction as per table below:

Adapter Ligation Master Mix

Component	Volume Per Individual Sample *inc. 10% excess*
Ligation Buffer	40 µL
DNA Ligase	10 µL
Total Adapter Ligation master mix volume	**50 µL**

2. Keeping the plate/tube(s) on ice, combine the 2nd strand synthesis product and diluted KAPA Adapter stock, then add the Adapter Ligation master mix as per the table below:

Component	Volume Per Individual Sample
2nd strand synthesis product	60 µL
Adapter ligation master mix	45 µL
Diluted adapter stock (*Chapter 2*)	5 µL
Total volume	**110 µL**

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

Ensure that the thermocycler lid temperature is set to +50°C.

4. Incubate the plate/tube(s) at +20°C for 15 min.
5. Proceed immediately to the next step.

To achieve higher ligation efficiency, particularly for difficult or degraded (i.e. FFPET-derived RNA) samples, consider increasing the ligation time to a maximum of 4 hrs at +20°C or overnight at +2°C to +8°C. Please note that longer ligation times may lead to increased levels of adapter-dimer. Adapter concentrations may have to be optimized if ligation times are extended significantly.

6. Proceed immediately to *Step 9. Purify the Sample Library using KAPA Pure Beads*.

Step 9. Purify the Sample Library using KAPA Pure Beads

If KAPA UDI Adapters were used for library construction – proceed to Step 9a or,

If KAPA Universal Adapter were used for library construction – proceed to Step 9b

Step 9a. Purify the Sample Library constructed using KAPA UDI Adapter (first cleanup)

Only perform this 0.63X cleanup if full length, KAPA UDI Adapters were used. Proceed to *Step 9b* if the KAPA Universal Adapter was used in order to perform a single 0.7X post-ligation cleanup.

1. To each ligation reaction, add 70 µL of room temperature KAPA Pure Beads that have been thoroughly resuspended as per the table below:

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

2. Once added, mix thoroughly and centrifuge briefly to collect all droplets. Do NOT allow beads to pellet.

   It is important at this step to ensure that the solution is thoroughly mixed and appears homogeneous. Insufficient mixing may compromise recovery and result in size selection.

3. Incubate the sample at room temperature for 5 min to bind DNA to the beads.
4. Place the sample on a magnet to capture the beads. Incubate until the liquid is clear.
5. Carefully remove and discard the supernatant.
6. Keeping the sample on the magnet, add 200 µL of 80% ethanol.
7. Incubate the sample on the magnet at room temperature for ≥30 sec.
8. Carefully remove and discard the ethanol.
9. Keeping the sample on the magnet, add 200 µL of 80% ethanol.
10. Incubate the sample on the magnet at room temperature for ≥30 sec.
11. Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.
12. Dry the beads at room temperature for 3 – 5 min, or until all of the ethanol has evaporated.

    Over-drying the beads may result in dramatic yield loss. Over-drying is indicated by a dry, cracked appearance of the bead pellet. The bead pellet should have a matte appearance when sufficiently dried.

13. Remove the sample from the magnet.
14. Thoroughly resuspend the beads in 50 µL of elution buffer (10 mM Tris-HCl, pH 8.0 – 8.5) to proceed with a 2nd post-ligation cleanup using PEG/NaCl *(Step 10. Purify the Sample Library using PEG/NaCl).*
15. Incubate the sample at room temperature for 2 minutes to allow the sample library to elute off the beads.

    Safe Stopping Point - If necessary this is a 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 *Step 10. Purify the Sample Library using PEG/NaCl*.

Step 9b. Purify the Sample Library constructed using KAPA Universal Adapter

Only perform this 0.7X cleanup if KAPA Universal Adapters were used. Refer to ***Step 9a*** if the KAPA UDI Adapters were used in order to perform a 0.63X post-ligation cleanup.

1. To each ligation reaction, add 77 µL of room temperature KAPA Pure Beads that have been thoroughly resuspended as per the table below:

Component	Volume Per Individual Sample
Adapter-ligated DNA	110 µL
KAPA Pure Beads	77 µL
Total volume	**187 µL**

2. Once added, mix thoroughly and centrifuge briefly to collect all droplets. Do **NOT** allow beads to pellet.

It is important at this step to ensure that the solution is thoroughly mixed and appears homogeneous. Insufficient mixing may compromise recovery and result in size selection.

3. Incubate the sample at room temperature for 5 min to bind DNA to the beads.
4. Place the sample on a magnet to capture the beads. Incubate until the liquid is clear.
5. Carefully remove and discard the supernatant.
6. Keeping the sample on the magnet, add 200 µL of 80% ethanol.
7. Incubate the sample on the magnet at room temperature for ≥30 sec.
8. Carefully remove and discard the ethanol.
9. Keeping the sample on the magnet, add 200 µL of 80% ethanol.
10. Incubate the sample on the magnet at room temperature for ≥30 sec.
11. Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.
12. Dry the beads at room temperature for 3 – 5 min, or until all of the ethanol has evaporated.

Over-drying the beads may result in dramatic yield loss. Over-drying is indicated by a dry, cracked appearance of the bead pellet. The bead pellet should have a matte appearance when sufficiently dried.

13. Remove the sample from the magnet.
14. Thoroughly resuspend the beads in 22 µL of 10 mM Tris-HCl, pH 8.0 – 8.5.
15. Incubate the sample at room temperature for 2 minutes to allow the sample library to elute off the beads.
16. Place the sample on a magnet to capture the beads. Incubate until the liquid is clear.
17. Transfer 20 µL of the clear supernatant to a new plate/tube(s) and proceed to ***Library Amplification (Chapter 4, Step 1)***.

Safe Stopping Point - If necessary, this is a safe stopping point. Purified, adapter-ligated library may be stored at +2°C to +8°C for 1 – 2 weeks or at -15°C to -25°C for ≤1 month before amplification. To avoid degradation, always store DNA in a buffered solution (10 mM Tris-HCl, pH 8.0 – 8.5) when possible, and minimize the number of freeze-thaw cycles.

Step 10. Purify the Sample Library using PEG/NaCl (second cleanup)

⚠ This is a **mandatory second cleanup** if KAPA UDI Adapters were used during library construction. Only perform this 0.7X cleanup if KAPA UDI Adapters were used. **SKIP** this step if KAPA Universal Adapters were used.

1. To each sample, add 35 µL of room temperature PEG/NaCl solution that have been thoroughly resuspended as per the table below:

Component	Volume Per Individual Sample
Beads with purified, adapter-ligated DNA (from *Step 9a*)	50 µL
PEG/NaCl Solution	35 µL
Total volume	**85 µL**

2. Once added, mix thoroughly and centrifuge briefly to collect all droplets. Do NOT allow beads to pellet.
3. Incubate the sample at room temperature for 5 min to bind DNA to the beads.
4. Place the sample on a magnet to capture the beads. Incubate until the liquid is clear.
5. Carefully remove and discard 80 µL of supernatant.
6. Keeping the sample on the magnet, add 200 µL of 80% ethanol.
7. Incubate the sample on the magnet at room temperature for ≥30 sec.
8. Carefully remove and discard the ethanol.
9. Keeping the sampleon the magnet, add 200 µL of 80% ethanol.
10. Incubate the sample on the magnet at room temperature for ≥30 sec.
11. Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.
12. Dry the beads at room temperature for 3 – 5 min, or until all of the ethanol has evaporated.

⚠ Over-drying the beads may result in dramatic yield loss. Over-drying is indicated by a dry, cracked appearance of the bead pellet. The bead pellet should have a matte appearance when sufficiently dried.

13. Remove the sample from the magnet.
14. Thoroughly resuspend the beads in 22 µL of 10 mM Tris-HCl (pH 8.0 – 8.5).
15. Incubate the sample at room temperature for 2 min to allow the sample library to elute off the beads.
16. Place the sample on a magnet to capture the beads. Incubate until the liquid is clear.
17. Transfer 20 µL of the clear supernatant to a new plate/tube(s) and proceed to *Library Amplification (Chapter 4, Step 1).*

Safe Stopping Point - If necessary, this is a safe stopping point. Purified, adapter-ligated library may be stored at +2°C to +8°C for 1 – 2 weeks or at -15°C to -25°C for ≤1 month before amplification. To avoid degradation, always store DNA in a buffered solution (10 mM Tris-HCl, pH 8.0 – 8.5) when possible, and minimize the number of freeze-thaw cycles.

Chapter 4.

Amplify the Sample Library

This chapter describes how to amplify the adapter-ligated library, using KAPA HiFi HotStart ReadyMix and appropriate compatible indexes or primer sets.

If KAPA UDI Adapters were used for adapter ligation, ensure KAPA Library Amplification Primer Mix (provided with the kit) is used for library amplification.

If KAPA Universal Adapters were used for adapter ligation, ensure a unique KAPA UDI Primer Mix is added to each sample library.

Ensure that the following is available:

- Freshly-prepared 80% ethanol
- 10 mM Tris-HCl, pH 8.0 – 8.5
- Nuclease-free, PCR-grade water

Library Amplification Considerations

- KAPA HiFi HotStart, the enzyme provided in the KAPA HiFi HotStart ReadyMix, is an antibody-based hotstart formulation of KAPA HiFi DNA Polymerase—a B-family DNA polymerase engineered for increased processivity and high fidelity. KAPA HiFi HotStart has 5'-->3' polymerase and 3'-->5' exonuclease (proofreading) activity, but no 5'-->3' exonuclease activity. The error rate of KAPA HiFi HotStart is 2.8 x 10^{-7} errors/base, equivalent to 1 error in 3.5 x 10^{6} nucleotides incorporated.
- The KAPA 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 the highest 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 DNA Polymerase). 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 can produce 8 – 10 µg of amplified library. To minimize over-amplification and its associated, undesired artifacts, the number of amplification cycles should be tailored to produce the optimal amount of final library required for downstream processes. This is typically in the range of 250 ng – 1.5 µg.

Step 1. Prepare the Library Amplification Reaction

Make sure the KAPA Pure Beads are removed from storage at least 30 minutes prior to starting the workflow to ensure they are equilibrated to room temperature. For best performance, store the beads protected from light when not in use.

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

If applicable, retrieve and thaw the KAPA UDI Primer Mixes plate prepared in *Chapter 2, Step 2c.*

Centrifuge the KAPA UDI Primer Mixes plate at 280 x *g* for 30 seconds to collect the contents to the bottom of the wells and peel off or pierce the foil seal for the appropriate number of wells needed. If only using a subset of the KAPA UDI Primer Mixes from the original plate, remove and discard residual primers from the well and apply a new adhesive foil seal provided in the kit.

If piercing the foil seal, avoid cross contamination by using a new pipette tip for every well.

Proper re-sealing and storage of the KAPA UDI Primer Mixes plate is necessary for unused primer mixes utilization at a later date.

Keep all components on ice as long as possible during handling.

1. Assemble each library amplification reaction as per table below:

Component	Volume per Individual Library
KAPA HiFi HotStart ReadyMix (2X)	25 µL
KAPA Library Amplification Primer Mix* OR KAPA UDI Primer Mix**	5 µL
Adapter-ligated library	20 µL
Total volume	**50 µL**

*If KAPA UDI Adapters were used for adapter ligation, ensure KAPA Library Amplification Primer Mix is used for library amplification. The KAPA HiFi HotStart ReadyMix and KAPA Library Amplification Primer Mix should preferably be premixed and added in a single pipetting step.

**If KAPA Universal Adapters were used for adapter ligation ensure a unique KAPA UDI Primer Mix is added to each sample library.

2. Mix thoroughly and centrifuge briefly. Immediately proceed to the next step.

Step 2. Perform the Library Amplification

1. Place the sample in the thermocycler and amplify the Sample library using the following Library Amplification program with the lid temperature set to +105°C:

Step	Temperature	Duration	Cycles
Initial denaturation	+98°C	45 sec	1
Denaturation	+98°C	15 sec	Variable, see Table 2 below
Annealing	+60°C	30 sec	
Extension	+72°C	30 sec	
Final extension	+72°C	1 min	1
Hold	+4°C	∞	1

Table 2: Recommended cycle numbers

Quantity of starting material	Number of cycles*
25 - 100 ng	11 – 15
101 - 250 ng	9 – 12
251 - 500 ng	7 – 10
501 - 1000 ng	6 – 8

*The recommended number of cycles is irrespective of which adapter kit was used during library construction (KAPA UDI Adapter or KAPA Universal Adapter). Note: The recommended number of cycles should be used as a guide for library amplification. Cycle numbers may require adjustment depending on RNA input quality, library amplification efficiency, presence of adapter-dimer, and the desired yield post amplification. Quantification of material after the second post-ligation cleanup using a qPCR assay, such as the KAPA Library Quantification Kit, can help to determine the number of amplification cycles required for a specific sample type or application.

2. Proceed immediately to the next step.

Step 3. Purify the Amplified Sample Library using KAPA Pure Beads

If KAPA UDI Adapters and KAPA Library Amplification Primer Mix were used for library construction – proceed to Step 3a or,

If KAPA Universal Adapter and KAPA UDI Primer Mixes were used for library construction – proceed to Step 3b.

Step 3a. Purify the Amplified Sample Library constructed using KAPA UDI Adapter & KAPA Library Amplification Primer Mix

1. Add 50 µL of room temperature, thoroughly resuspended, KAPA Pure Beads to each amplified sample library.
2. Mix the amplified sample library and KAPA Pure Beads thoroughly and centrifuge briefly to collect all droplets. Do **NOT** allow beads to pellet.
3. Incubate the sample at room temperature for 5 minutes to allow the amplified sample library to bind to the beads.
4. Place the sample on a magnet to capture the beads. Incubate until the liquid is clear.
5. Carefully remove and discard the supernatant.
6. Keeping the sample on the magnet, add 200 µL of freshly-prepared 80% ethanol.
7. Incubate the sample at room temperature for ≥30 seconds.
8. Carefully remove and discard the ethanol.
9. Keeping the sample on the magnet, add 200 µL of freshly-prepared 80% ethanol.
10. Incubate the sample at room temperature for ≥30 seconds.
11. Carefully remove and discard the ethanol. Remove residual ethanol without disturbing the beads.
12. Allow the beads to dry at room temperature, sufficiently for all of the ethanol to evaporate.

    Over-drying the beads may result in dramatic yield loss. Over-drying is indicated by a dry, cracked appearance of the bead pellet. The bead pellet should have a matte appearance when sufficiently dried.

13. Remove the sample from the magnet.
14. Thoroughly resuspend the beads in 22 µL (or appropriate volume) of 10 mM Tris-HCl, pH 8.0 – 8.5.
15. Incubate the sample at room temperature for 2 minutes to allow the amplified sample library to elute off the beads.
16. Place the sample on the magnet to capture the beads. Incubate until the liquid is clear.
17. Transfer the clear supernatant to a new tube(s)/well and proceed with library QC (*Chapter 5. Quality Control*), target capture or sequencing, as appropriate.
18. Purified, amplified sample libraries can be stored at +2°C to +8°C for 1 - 2 weeks or at -15°C to -25°C for up to 3 months.

Step 3b. Purify the Amplified Sample Library constructed using KAPA Universal Adapter & KAPA UDI Primer Mixes

A modified post-amplification cleanup is highly recommended when using KAPA Universal Adapter & KAPA UDI Primer Mixes with KAPA library preparation kits. Especially if libraries will be sequencing on an Illumina NovaSeq or HiSeq X system (with patterned flow cells). This additional or modified cleanup will serve to remove any indexed primer carryover which may exacerbate index hopping.

The KAPA Pure Beads provided with the 24 rxn and 96 rxn pack size, may not be sufficient if processing 24 or 96 samples with the KAPA Universal Adapter & KAPA UDI Primer Mixes. Additional KAPA Pure Beads is sold separately. Alternatively, the unused PEG/NaCl provided with the kit may be repurposed for a "with-bead" protocol. Please contact *Technical Support* for guidance.

1. Add 50 µL of room temperature, thoroughly resuspended, KAPA Pure Beads to each amplified sample library.
2. Mix the amplified sample library and KAPA Pure Beads thoroughly and centrifuge briefly to collect all droplets.
3. Incubate the sample at room temperature for 5 minutes to allow the amplified sample library to bind to the beads.
4. Place the sample on a magnet to capture the beads. Incubate until the liquid is clear.
5. Carefully remove and discard the supernatant.
6. Remove the tubes from the magnet, and resuspend the beads in 50 µL of nuclease-free PCR-grade water or 10 mM Tris-HCl, pH 8.0 – 8.5.
7. Add 50 µL of KAPA Pure Beads to each sample.
8. Mix thoroughly by pipetting or vortexing, and centrifuge briefly.
9. Incubate the sample at room temperature for 5 minutes to allow the amplified sample library to bind to the beads.
10. Place the sample on a magnet to capture the beads. Incubate until the liquid is clear.
11. Carefully remove and discard the supernatant.
12. Keeping the sample on the magnet, add 200 µL of freshly-prepared 80% ethanol.
13. Incubate the sample at room temperature for ≥30 seconds.
14. Carefully remove and discard the ethanol.
15. Keeping the sample on the magnet, add 200 µL of freshly-prepared 80% ethanol.
16. Incubate the sample at room temperature for ≥30 seconds.
17. Carefully remove and discard the ethanol. Remove residual ethanol without disturbing the beads.
18. Allow the beads to dry at room temperature, sufficiently for all of the ethanol to evaporate.

    Over-drying the beads may result in dramatic yield loss. Over-drying is indicated by a dry, cracked appearance of the bead pellet. The bead pellet should have a matte appearance when sufficiently dried.

19. Remove the sample from the magnet.
20. Thoroughly resuspend the beads in 22 µL (or appropriate volume) of 10 mM Tris-HCl, pH 8.0 - 8.5.
21. Incubate the sample at room temperature for 2 minutes to allow the sample library to elute off the beads.
22. Place the sample on the magnet to capture the beads. Incubate until the liquid is clear.
23. Transfer the clear supernatant to a new tube(s)/well and proceed with library QC (*Chapter 5. Quality Control*), target capture or sequencing, as appropriate.
24. Purified, amplified sample libraries can be stored at +2°C to +8°C for 1 - 2 weeks or at -15°C to -25°C for up to 3 months.

Chapter 5.

Quality Control

This chapter describes how to determine the concentration and size distribution of the sample library. 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 the next step in the process (e.g., target capture or sequencing), as well as for library QC and archiving purposes.

Quantification

KAPA Library Quantification Kits for Illumina platforms are recommended for qPCR-based quantification of libraries generated using the KAPA RNA HyperPrep Kit with RiboErase (HMR) workflow. Libraries may also be quantified using a Qubit Fluorometer and the Qubit dsDNA HS Assay Kit.

Sizing

The size distribution of the final libraries should be confirmed with an electrophoretic method. A LabChip GX, GXII, or GX Touch (PerkinElmer), Bioanalyzer, TapeStation (Agilent Technologies), Fragment Analyzer (Advanced Analytical) system or similar instruments are recommended over conventional gels. Libraries generated using the KAPA RNA HyperPrep Kit with RiboErase (HMR) may require dilution prior to electrophoretic assessment. Refer to the respective instrument and assay user manuals for guidance on how to dilute your sample library so as not to exceed the detection limit of the specific assay.

Appendices

Appendix A. Library Size Distribution Optimization

The KAPA RNA HyperPrep Kit with RiboErase (HMR) offers a tunable RNA fragmentation module in which RNA is fragmented at a high temperature in the presence of magnesium. Final library size distributions can be optimized for specific sample types and applications by varying both incubation time and temperature. Generally:

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

Fragmentation times may require adjustment based upon the quality of the input RNA. For intact RNA, such as that extracted from fresh/frozen tissue, longer fragmentation is required at higher temperatures. For degraded or fragmented RNA (e.g., from older samples or FFPE tissue), a lower temperature and/or shorter time should be used.

Intact Total RNA Inputs

When using high-quality, intact total RNA, the following recommendations can be used as a starting point for the optimization of final library distributions beyond what is provided within *Chapter 3*. It is recommended that a non-precious, representative RNA sample be used for this optimization. For the following figures and tables, final libraries were generated using 100 ng of high-quality Universal Human Reference (UHR) RNA.

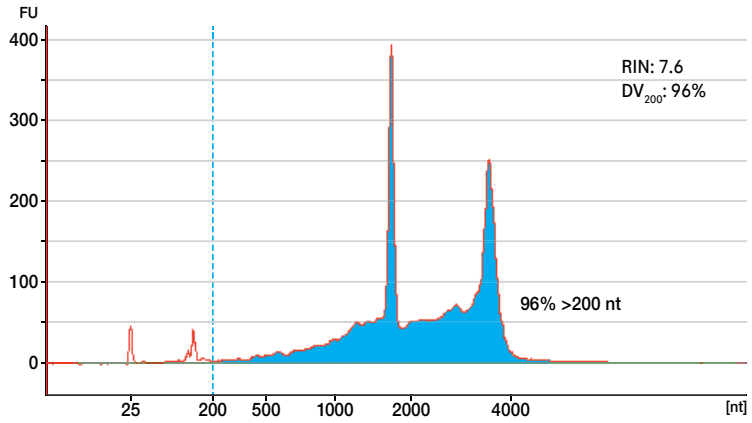


Figure 3. High-quality UHR total RNA electropherogram prior to library construction
RIN score was 7.6, with 96% of the RNA measuring >200 nucleotides, (i.e. the DV_{200} score) as assessed via an Agilent RNA 6000 Pico Kit.

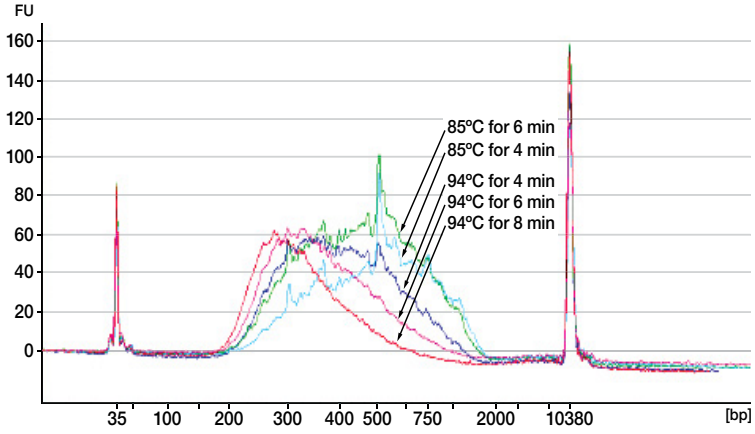


Figure 4. Final library distributions demonstrating fragmentation tunability.
Higher temperatures and longer incubation times resulted in shorter, narrower final library distributions. Libraries were constructed using 100 ng of high-quality UHR RNA and various fragmentation conditions. Note that results may differ with other sample sources. Electropherograms were generated with an Agilent High Sensitivity DNA Kit.

Table 3. Approximate mean and mode final library sizes (bp) for each fragmentation condition assessed

Fragmentation	Final library size (bp)	
	Mean	Mode
+94°C for 8 min	~350	~280
+94°C for 6 min	~380	~320
+94°C for 4 min	~440	~370
+94°C for 2 min	~550	~500
+85°C for 6 min	~510	~510
+85°C for 4 min	~570	~510

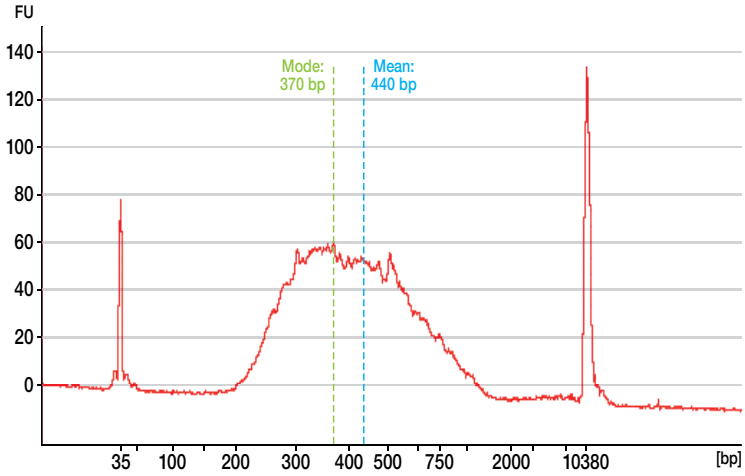
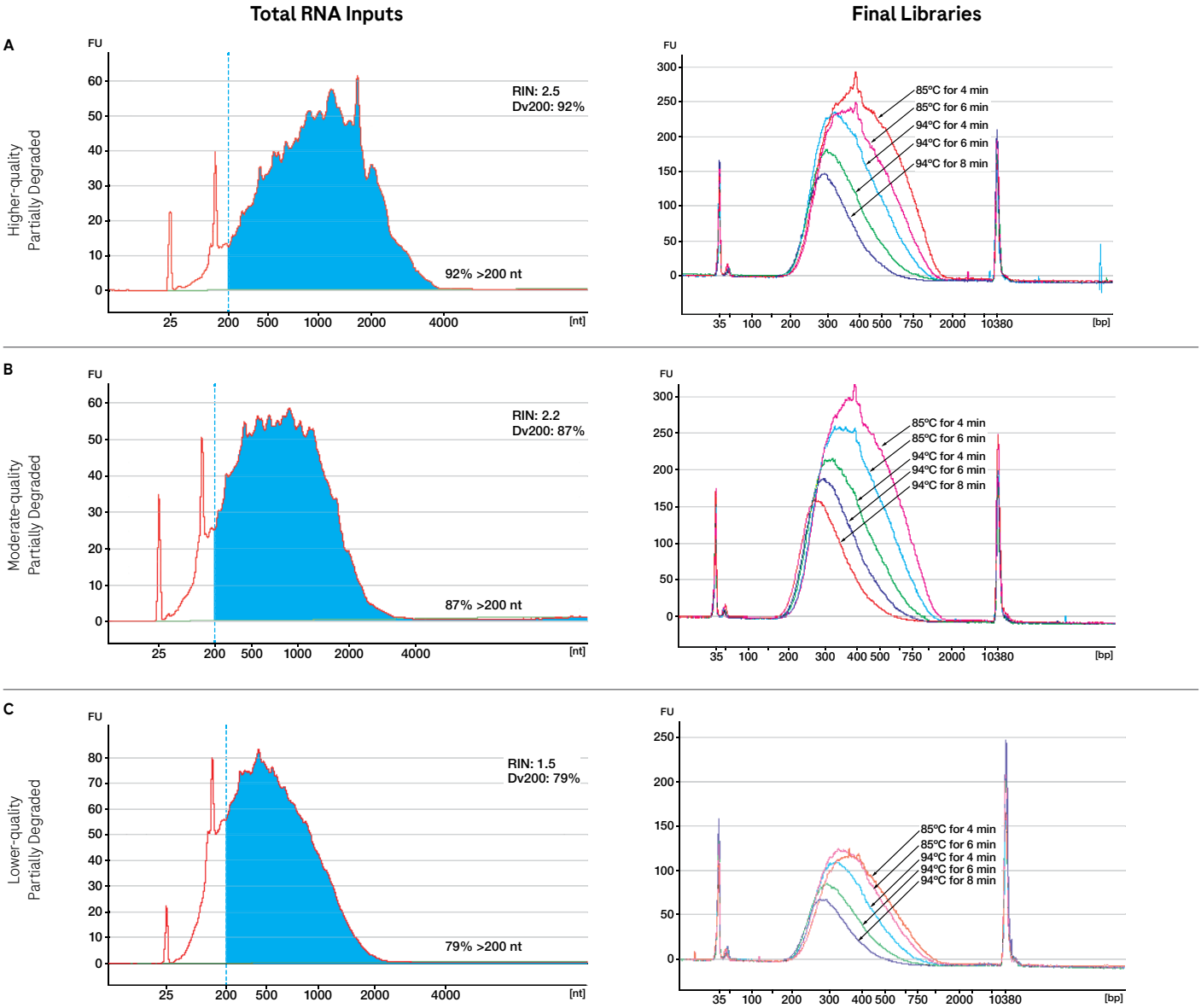


Figure 5. Visual depiction of the mean and mode distribution metrics.
For a final library generated using 100 ng UHR fragmented at +94°C for 4 minutes, the mode is the highest peak in the library (~370 bp), while the mean is the numerical average across all molecular lengths in the library (~440 bp). In this example, the mean of the library is calculated across the range of 190 to 1600 bp. The higher molecular weight shoulder of the distribution results in the mean being larger than the mode.

Partially Degraded Total RNA Inputs

When working with partially degraded (PD) inputs, the following recommendations may serve as a starting point for the optimization of final library distributions beyond what is provided within *Chapter 3.* It is recommended that a non-precious, representative RNA sample be used for optimization. For the following figures and tables, chemically degraded samples of UHR were used as substitutes for real-world partially degraded samples of varying qualities. This RNA was ribosomally-depleted using the KAPA RiboErase (HMR) RNA enrichment module, processed with the KAPA RNA HyperPrep workflow, and subjected to a fragmentation condition titration.



Fragmentation	Higher-quality Partially Degraded Final Library Size		Moderate-quality Partially Degraded Final Library Size		Lower-quality Partially Degraded Final Library Size	
	Mean (bp)	Mode (bp)	Mean (bp)	Mode (bp)	Mean (bp)	Mode (bp)
+94°C for 8 min	~320	~280	~310	~270	~310	~280
+94°C for 6 min	~350	~300	~340	~300	~330	~290
+94°C for 4 min	~380	~330	~380	~310	~360	~310
+85°C for 6 min	~420	~390	~410	~330	~390	~330
+85°C for 4 min	~450	~390	~450	~390	~410	~360

Figure 6. Input RNA and final library distributions for a range of partially-degraded sample qualities.

Libraries were constructed using 100 ng of chemically-degraded UHR RNA to target various qualities of partially degraded inputs, including higher-quality (A), moderate-quality (B), and lower-quality (C). As expected, the two commonly used RNA quality metrics, RIN and Dv_{200} decrease as RNA quality decreases. For all RNA qualities assessed, increased fragmentation time and/or temperature resulted in shorter, narrower distributions. Note that results may differ with other sample sources. Total RNA electropherograms were generated with an Agilent RNA 6000 Pico Kit, and final library electropherograms were generated with an Agilent 2100 High Sensitivity DNA Kit.

The effects of total RNA quality on final library distributions are illustrated in *Figure 7*, and summarized below by fragmentation condition:

- +94°C for 8 min: Final library distributions were not overly impacted by input RNA quality, but a correlation between lower quality and reduced final library yield was apparent.
- +94°C for 4 min: While final library distribution modes were not overly impacted by input RNA quality, distributions became narrower, resulting in lower mean values, as RNA quality decreased.
- +85°C for 4 min: Both the mode and mean final library distribution metrics were impacted by RNA input quality, with both metrics decreasing as input quality decreased. Final library yield was not overly impacted until input quality dropped sufficiently low.

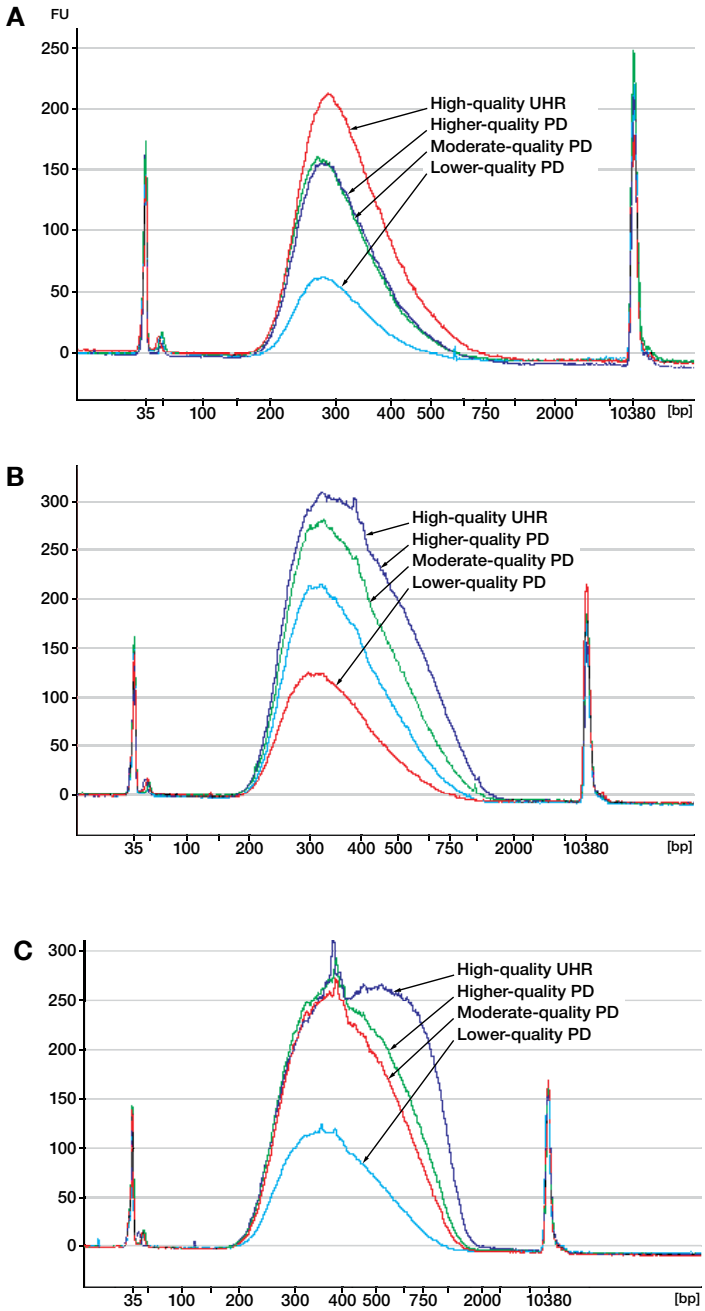


Figure 7. Assessment of the effects of total RNA quality on final library distributions.

Fragmentation conditions were elected to target a range of final library sizes: +94°C for 8 min (A), +94°C for 4 min (B), and +85°C for 4 min (C). Libraries were constructed using 100 ng of either intact UHR RNA (UHR) or partially-degraded (PD) UHR RNA. Results may differ with other sample sources. Electropherograms were generated with an Agilent High Sensitivity DNA Kit.

FFPE-derived Total RNA Inputs

When working with FFPE-derived total RNA inputs, fragmentation at +65°C for 1 min is recommended. In the following figures and table, final libraries were generated using two FFPE-derived samples, one of higher-quality and one of lower-quality. Total RNA was ribosomally-depleted using the KAPA RiboErase (HMR) RNA enrichment module and then processed with the KAPA RNA HyperPrep workflow.

It should be noted that variable qualities of FFPE-derived samples can impact both final library size distributions and the amount of adapter-dimer carryover. In the case of elevated amounts of residual adapter-dimer, perform a second post-amplification 1X KAPA Pure Beads cleanup. Adapter-dimer carryover can be prevented in future library preparations by reducing the adapter concentration in the ligation reaction.

Table 4. Approximate final mean and mode library sizes, in bp, and adapter-dimer carryover rate for higher- and lower-quality FFPE samples.

Sample	Final library size (bp)		Adapter-dimer (Molar %)
	Mean	Mode	
Thyroid (higher-quality)	~350	~310	0.8
Duodenum (lower-quality)	~300	~280	14.2

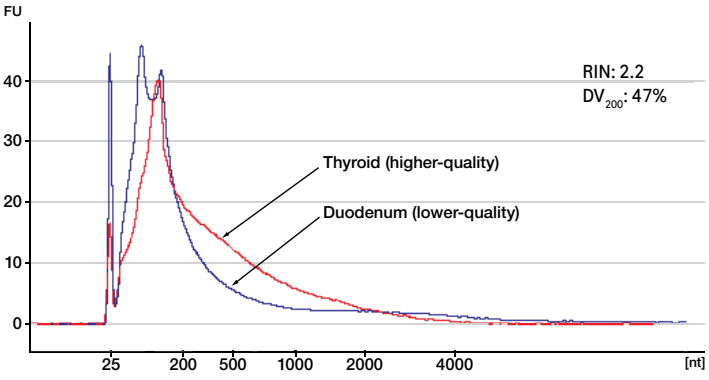


Figure 8. Electropherograms for total RNA inputs derived from thyroid and duodenum FFPE samples.
The high-quality thyroid sample had a RIN score of 2.2, with 47% of the RNA measuring >200 nucleotides. The lower-quality duodenum sample had a RIN score of 2.5, with 29% of the RNA measuring >200 nucleotides (i.e. DV_{200} = 29%). Both quality metrics were assessed via an Agilent RNA 6000 Pico Kit.

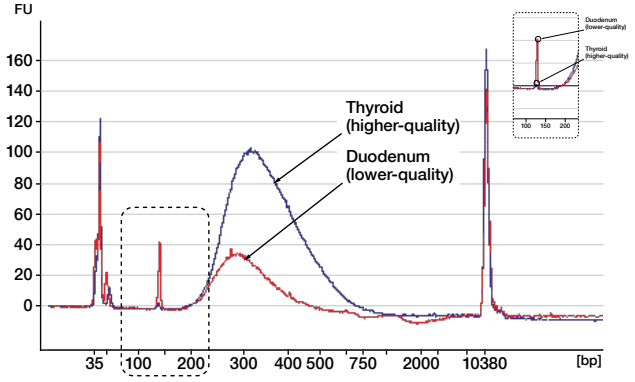


Figure 9. Final libraries resulting from FFPE-derived RNA inputs.
The lower-quality FFPE input showed a slightly smaller size distribution and a higher prevalence of adapter-dimer in comparison to the higher-quality FFPE input. Libraries were constructed using 100 ng of total RNA fragmented at +65°C for 1 minute. Electropherograms were generated with an Agilent High Sensitivity DNA Kit.

Appendix B. Limited Warranty

1. Limited Warranty

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