Application Note

Whole Transcriptome Sequencing from FFPE-derived RNA

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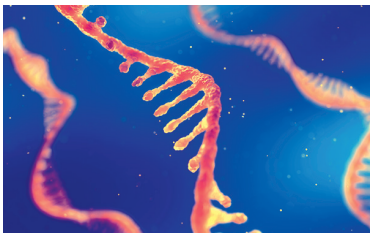
KAPA RNA HyperPrep Kits with RiboErase (HMR) offer a reliable, single-day library prep solution for whole transcriptome sequencing from FFPE-derived RNA

High-resolution RNA analysis using next-generation sequencing (RNA-Seq) is enabling advances in translational and clinical research. The quality of RNA extracted from biological specimens can be highly variable, and quantities limiting; thereby impacting the ability to generate high-quality sequencing libraries. KAPA RNA HyperPrep Kits with RiboErase (HMR) support reliable whole transcriptome sequencing from degraded inputs.

Introduction



Formalin-fixed paraffin-embedded (FFPE) tissue is a valuable resource for translational and clinical research. The quality of RNA extracted from FFPE tissue can, however, be highly variable due to the damaging nature of the fixation process (which may result in crosslinking, chemical modification, and fragmentation), the age of the tissue, and the RNA extraction method used. Efficient library preparation holds the key to unlocking the potential of FFPE samples for RNA-Seq. It is important to select the appropriate library construction technology, understand the potential impact of RNA quality on library and data quality, and tailor key library prep and sequencing parameters accordingly.

Libraries for RNA-Seq on the Illumina platform may be constructed using several different strategies. The preparation of total RNA libraries requires the least amount of input material, and enables the most comprehensive view of the transcriptome. However, to increase sequencing economy, and improve coverage of low-abundance transcripts of interest, undesired content may either be removed prior to library construction; or desired content may be selected from total cDNA libraries after library construction. The elimination of ribosomal RNA (rRNA; which constitutes ~90% of total RNA) prior to library construction is a widely used approach. This is achieved by enrichment for messenger RNA (mRNA capture), or the depletion of rRNA.

KAPA RiboErase (HMR) Kits are designed for the enzymatic (RNase H-based) depletion of unwanted rRNA transcripts before library construction. Unlike mRNA capture, this technology does not target the poly-A tails of mature mRNAs. It is therefore highly suitable for library construction from FFPE-derived or other degraded RNA inputs, and does not result in the strong bias for 3'-ends of transcripts that occurs during mRNA capture of low-quality inputs.

In this study, we prepared libraries from FFPE-derived RNA of variable quality to characterize the impact of RNA quality on library and sequencing data quality. Results confirm that the KAPA RNA HyperPrep Kit with RiboErase (HMR) enables reproducible and reliable whole transcriptome sequencing from degraded inputs. In addition, this Application Note provides insights for managing expectations when working with low-quality samples, and tools for optimizing the library preparation workflow.

Experimental Design and Methods

Library construction workflow

KAPA RNA HyperPrep Kits with RiboErase (HMR) are designed for the construction of high-quality libraries for whole transcriptome sequencing on the Illumina platform, from total RNA inputs ranging from 25 ng – 1 µg. The protocol consists of the following steps: (i) enzymatic depletion of rRNA transcripts, (ii) heat fragmentation of RNA and random priming, (iii) first-strand cDNA synthesis, (iv) second-strand synthesis and A-tailing, (v) the addition of Illumina-specific adapters, and (vi) library amplification. Enzymatic depletion entails the hybridization of DNA probes to cytoplasmic (5S, 5.8S, 18S, and 28S) and mitochondrial (12S and 16S) rRNA transcripts, followed by the degradation of RNA:DNA hybrids with RNase H. The entire workflow (Figure 1) is automation-friendly, and can be completed in a standard workday. rRNA depletion probes included in the kit are optimized for human, mouse and rat species. Additional probes for the simultaneous depletion of globin transcripts from blood samples are also available,[1] and the protocol may be adapted to provide for the depletion of other transcripts, using user-supplied probes.[2]

The validated KAPA RNA HyperPrep with RiboErase (HMR) protocol has built-in flexibility to cater for the wide range of input amounts and sample types that may be processed with the kit. Fragmentation parameters are tunable to best match the quality of the input material, and adapter concentration is tailored for different input amounts, to ensure optimal conversion rates. Likewise, the number of library amplification cycles is varied based on input, to avoid excessive PCR duplication.[3] Throughout the library construction process, quality control (QC) data may be collected to inform protocol decisions, and evaluate the success of library construction prior to sequencing. Recommended QC steps/assays are highlighted in Figure 1.

In addition to demonstrating the utility of the KAPA RNA HyperPrep Kit with RiboErase (HMR) for reliable whole transcriptome sequencing from FFPE-derived RNA, a primary objective of this study was to characterize the impact of RNA quality on the outcome of library construction and sequencing data quality. Insights presented here were distilled into an optimized protocol for library construction from low-quality RNA extracts, which may be found in an accompanying Technical Note entitled *How To… Prepare libraries from degraded RNA inputs with the KAPA RNA HyperPrep Kit with RiboErase (HMR) for whole transcriptome sequencing.*[4]

RNA samples

RNA derived from three different tissue types (breast tumor, duodenum and thyroid) were used in this study. For the breast tumor, RNA was extracted from matched fresh frozen (FF) and FFPE tissue samples of the same specimen. For duodenum and thyroid, RNA extractions were from FFPE tissue only. All extractions included a DNase I-treatment; and several extractions were performed from each tissue type, to generate a collection of samples with different quality profiles. For contrast, a high-quality commercial RNA preparation (Universal Human Reference, UHR; Agilent Technologies) was included in some experiments.

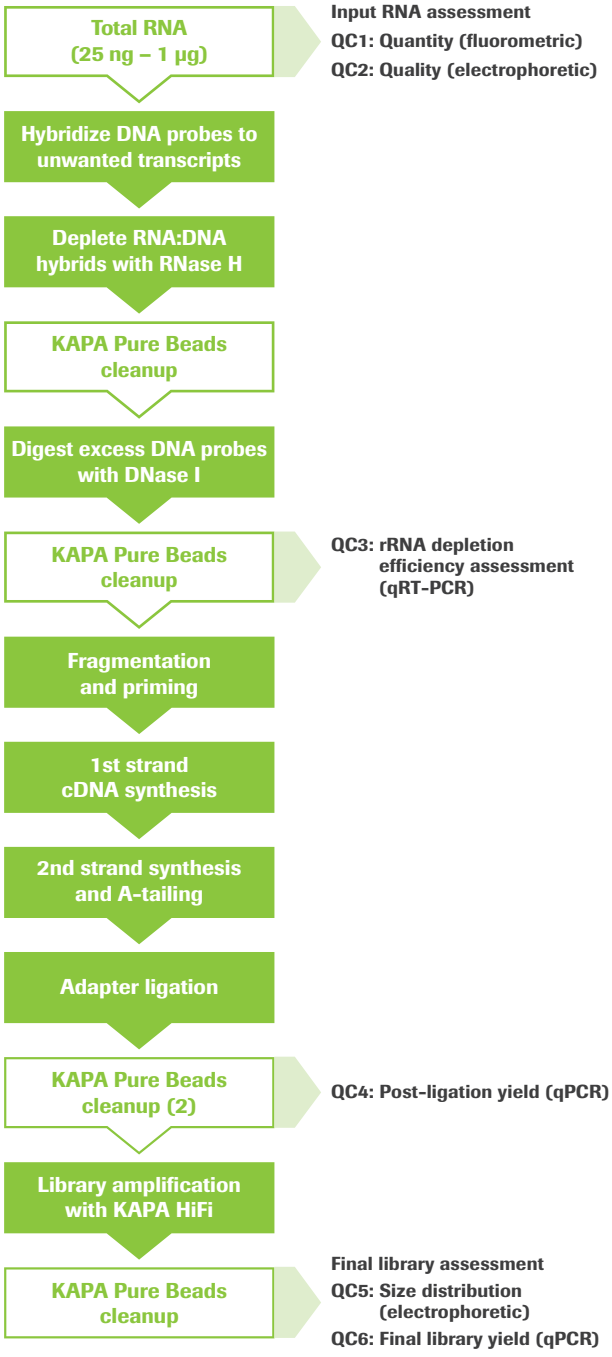


Figure 1: The KAPA RNA HyperPrep with RiboErase (HMR) workflow. The protocol, designed for the construction of libraries for whole transcriptome sequencing on the Illumina platform, requires 25 ng – 1 µg total RNA as input. The depletion process involves the hybridization of DNA probes to undesired transcripts, and two enzymatic degradations, each followed by a bead-based cleanup step. The library construction protocol has been significantly streamlined, to allow for completion of the entire process from input RNA to sequencing-ready library in a single workday (~6.5 hours). The kit contains all of the required reagents (including KAPA Pure Beads for reaction cleanups), with the exception of adapters, which are available separately from Roche.

Quality control (QC) points are highlighted, and the type of assay recommended in each case indicated in parentheses. In this study, RNA samples were quantified (QC1) using the Qubit RNA HS Assay (ThermoFisher Scientific). The Agilent Bioanalyzer 2100 System was used for all electrophoretic analyses; in combination with the Agilent RNA 6000 Pico Kit for RNA quality assessment (QC2), or the Agilent High Sensitivity DNA Kit for the analysis of library material (QC5). The KAPA Library Quantification Kit was used for the qPCR-based quantification of post-ligation (QC4) and final (QC6) libraries. rRNA depletion was assessed with an in-house developed qRT-PCR assay, with primers targeting human 28S rRNA and GAPDH transcripts, respectively. More details of these QC steps, as well as a detailed protocol for the qRT-PCR assay may be found in an accompanying Technical Note.[4]

Data on file.

Electrophoretic profiles for representative RNA samples from the collection are given in Figure 2. Two QC metrics for RNA quality, namely an RNA Integrity Number (RIN) and DV_{200} value, are provided for each sample. The RIN is calculated from the size distribution and relative ratios of rRNA peaks, and is automatically tabulated by the Agilent Expert software. Samples with a RIN ≥7 are considered to be of high quality. FFPE-extracted RNA typically lacks distinctive ribosomal peaks, thus impacting the relevance of the RIN as a quality metric for such samples. The DV_{200} value quantifies the percentage of the sample RNA with a fragment size greater than 200 nucleotides (nt) in length. RNA fragments shorter than 200 nt are poor substrates for RNA-Seq library construction and are likely to be lost during fragmentation, random priming and subsequent cleanup steps in the library prep workflow. In our experience, the DV_{200} value provides a better measure of RNA quality than the RIN when applied to lower-quality, degraded inputs.

While RINs suggested that all RNA extracts were of similarly low quality, DV_{200} values provided better resolution. These indicated the RNA from the fresh-frozen breast tumor tissue (Figure 2B; DV_{200} = 74%) and the thyroid FFPE tissue (Figure 2C; DV_{200} = 47%) to be of higher quality than the extract from the duodenum FFPE tissue (Figure 2D; DV_{200} = 29%). The RNA from breast tumor FFPE tissue (Figure 2E) also returned a high DV_{200} value (76%), but this value was likely artificially inflated by the high-molecular weight peak observed in this otherwise highly degraded sample. This peak was assumed to correspond to cross-linked or incompletely deparrafinized material, rather than actual intact RNA that could be utilized for library construction.

Library construction and QC

Replicate libraries were prepared with the KAPA RNA HyperPrep Kit, either from total RNA, or after depletion of rRNA with the KAPA RiboErase (HMR) Kit (Figure 1). QC data were generated at different stages of the process, as outlined in Figure 1. Post-ligation library yields were used to determine the number of amplification cycles needed to generate libraries with a final concentration of ≥10 nM, which is commonly recommended for the long-term storage of Illumina libraries. Unless otherwise indicated, all library prep and QC protocols were executed according to manufacturer's instructions. Negative (no template) controls were included in all qPCR-based assays. Final library pools were quantified with the KAPA Library Quantification Kit.

Sequencing and data analysis

Paired-end sequencing (2 x 100 bp) was performed on an Illumina HiSeq 2500 instrument, using a HiSeq v4 chemistry kit (Illumina). Adapter and quality trimming was performed using cutadapt and trimmomatic, respectively. Reads were aligned to a hard-masked version of human reference GRCh38, filtered to remove rRNA reads, and sub-sampled to the lowest common number of paired reads (14 million per sample). Gene expression was quantified and TMM-normalized using Kallisto (0.42.4).

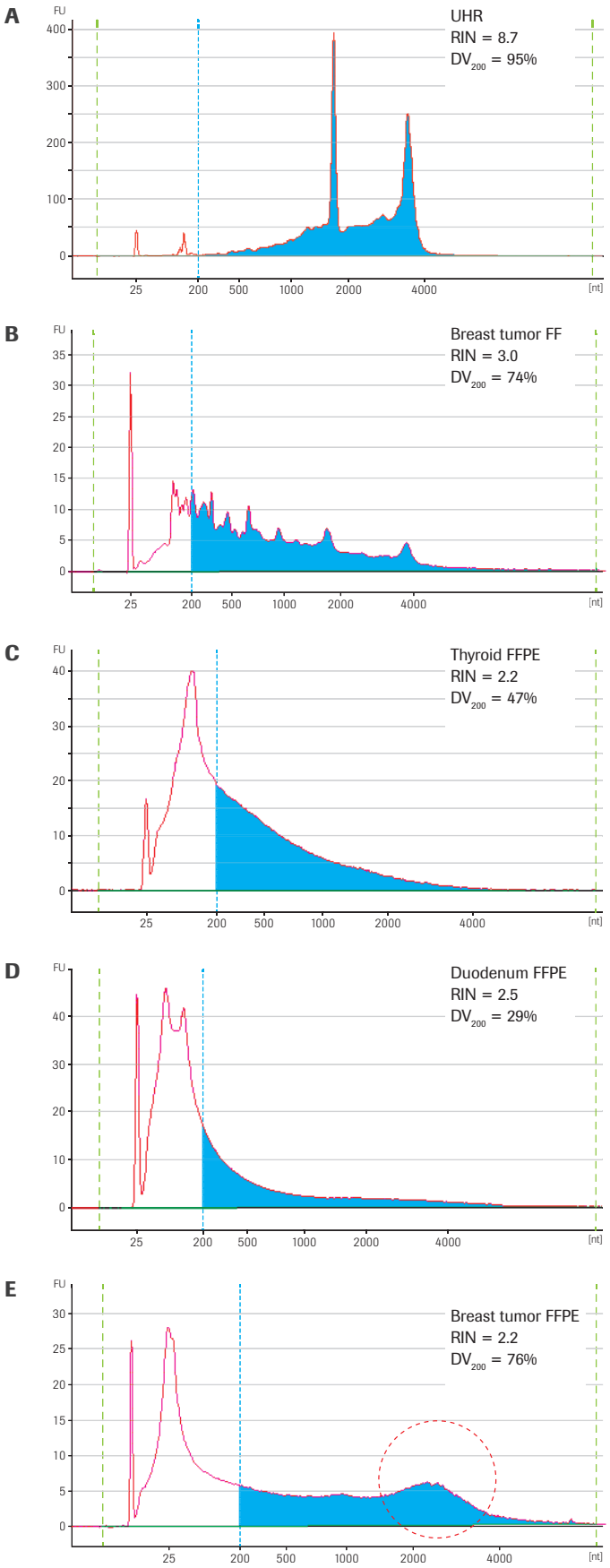


Figure 2: Electrophoretic profiles for representative RNA samples utilized in this study. Samples **C** – **E** were isolated from FFPE tissue; whereas sample **B** originated from fresh-frozen (FF) tissue of the same biological specimen as sample **C**. High-quality human Universal Human Reference RNA (Agilent Technologies; **A**) is included for comparison. Electropherograms were generated using an Agilent 2100 Bioanalyzer and RNA 6000 Pico Kit. Blue shading highlights RNA fragments >200 nt. The region circled in red in the breast tumor FFPE sample (**E**) designates a high-molecular weight peak that is likely the result of crosslinking or inefficient deparrafinization (rather than intact transcripts that could be efficiently converted to sequenceable cDNA fragments).

Results

Library yields and quality decrease as a function of RNA quality

In our first experiment aimed at assessing the impact of RNA quality on the outcome of library construction, total RNA libraries were prepared with the KAPA RNA HyperPrep Kit (without upfront rRNA depletion). Not surprisingly, results confirmed final library yields (from the same input amount) to decrease as a function of RNA quality (Figure 3).

Further investigation confirmed this trend to also apply to **post-ligation** library yields (before library amplification), which are a better indication of the efficiency of the core library construction process (Figure 4A). In addition, the concentration of adapter-dimers were found to increase with decreasing input RNA quality (Figure 4B). Adapter-dimers impair library quality and have to be removed prior to sequencing to ensure optimal clustering, particularly on patterned flow cells.[5]

Ligation reaction parameters can be optimized to improve the quality of libraries prepared from low-quality samples

From past experience with the optimization of DNA library preparation chemistries and protocols, we know that adapter-insert molar ratios play an important role in the efficiency of ligation-based library construction. Higher adapter-insert molar ratios typically result in improved conversion of DNA (or cDNA) fragments to sequenceable library molecules, particularly for low-input and/or low-quality samples. However, this benefit may be offset by an increased rate of adapter-dimer formation. Another key factor that impacts final library yield and composition (sequenceable molecules vs adapter-dimers) are the bead ratio(s) used for post-ligation cleanups.[6]

To assess the impact of modifying key parameters of the adapter ligation step on library quality, libraries were construted from a highly degraded FFPE-derived RNA sample (DV_{200} = 29%), using varying concentrations of adapter, and different post-ligation bead cleanup ratios. As expected, post-ligation yield improved with increased adapter concentration and less stringent post-ligation cleanups (Figure 5A). However, high apparent library yields were largely the result of unacceptable levels of adapter-dimer (Figure 5B). For this RNA sample, decreasing the adapter stock concentration by half (to 750 nM, from the standard concentration of 1.5 µM) and relaxing post-ligation cleanup ratios to 0.8X and 0.9X (from the standard 0.63X and 0.7X) reduced adapter-dimer formation without significantly decreasing library yield (when compared to the standard protocol; Figure 5C).

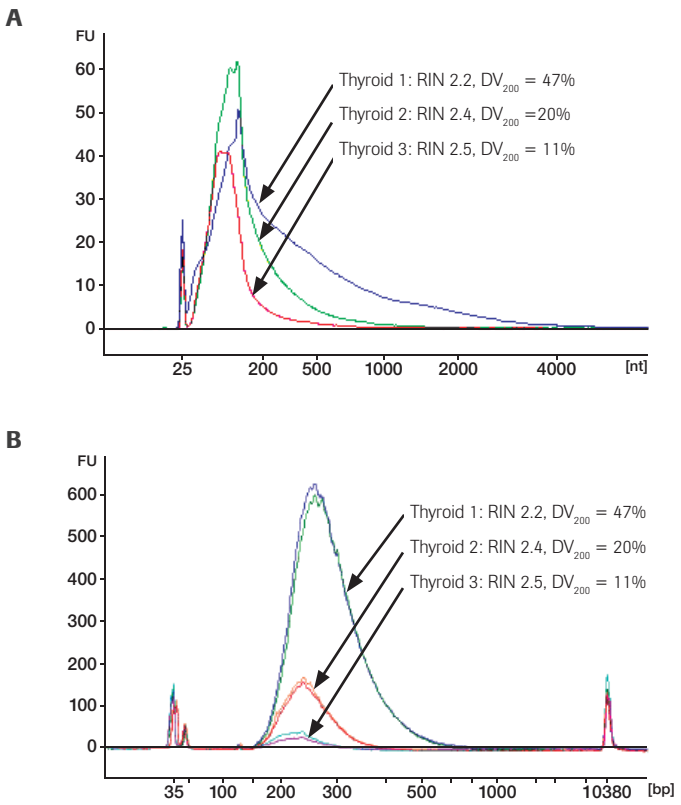


Figure 3: Final library yields decrease as a function of input RNA quality. Electrophoretic profiles for **(A)** three separate RNA extractions from thyroid FFPE tissue, and **(B)** final, total RNA-Seq libraries prepared from each extraction. Duplicate libraries were prepared from each extract, from 25 ng total RNA inputs, using the KAPA RNA HyperPrep Kit (with no upfront RNA enrichment). Libraries prepared from Thyroid 3 sample (DV_{200} value = 11%) were considered to not be worth sequencing.

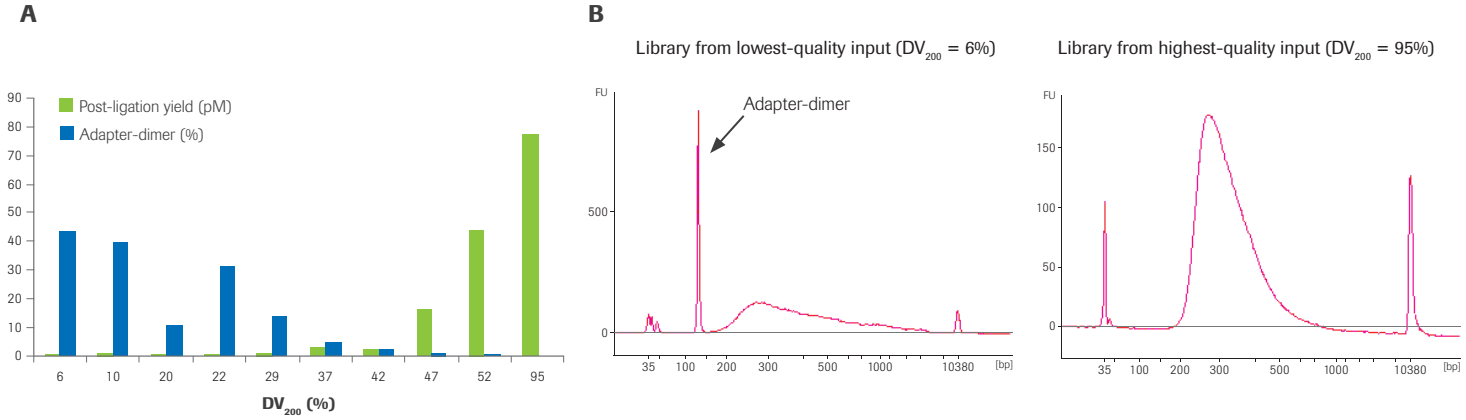


Figure 4: Post-ligation library yields and library quality decrease as a function of input RNA quality. Libraries were constructed with the KAPA RNA HyperPrep Kit with RiboErase (HMR), from 100 ng inputs of FFPE-derived RNA samples of variable quality (DV_{200} range: 6 – 52%). The high-quality UHR control included in the experiment had a DV_{200} value of 95%. **(A)** Post-ligation yields were measured with the KAPA Library Quantification Kit. Adapter-dimer rates were calculated from the electrophoretic assessment of final libraries. **(B)** Adapter-dimers are efficiently amplified (during library amplification and cluster generation), as demonstrated by the distinct peak at ~120 bp in the electrophoretic profile of the final library generated from the lowest-quality (DV_{200} = 6%) sample. This adapter-dimer peak is not detectable in the electrophoretic profile of the final library prepared from the high-quality (DV_{200} = 95%) control UHR sample.

Data on file.

Based on these observations, the KAPA RNA HyperPrep with RiboErase (HMR) protocol was optimized to improve outcomes for degraded RNA samples (DV_{200} value <50%). Please refer to the previously mentioned Technical Note for full details.[4]

Some, but not all key sequencing QC metrics are impacted by RNA quality

To assess the impact of RNA quality on sequencing data quality, triplicate libraries were prepared from different inputs (25 ng and/or 100 ng) of a subset of the RNA extracts prepared for this study. RNA extracted from the fresh frozen (FF) breast tumor tissue, and UHR RNA were also included in this experiment. All libraries were prepared with the KAPA RNA HyperPrep Kit with RiboErase (HMR), using the standard adapter stock concentration (1.5 µM) and post-ligation cleanup ratios (0.63X, 0.7X). Optimized protocols were intentionally not employed, as the aim was to identify data trends, rather than achieve the best result for each sample.

Input and library construction metrics are given in Table 1. Sequencing was performed as described in **Experimental Design and Methods**. Standard fragmentation conditions were employed for the UHR and FF samples, whereas both the fragmentation time and temperature were reduced for FFPE samples.[3,4] In terms of library QC metrics, pre- and post-amplification library yields, and adapter-dimer rates (derived from sequencing data, rather than electrophoretic analysis) were recorded. The following key sequencing QC metrics were obtained from secondary data analysis, and compared for the different libraries: percentage of mapped reads, the percentage of reads mapping to residual rRNA, the percentage of duplicate reads, coverage balance, and the number of unique transcripts identified for each sample. Library and sequencing QC data are summarized in Table 1 and Figure 6 on the next page.

As expected from the preceding experiments, libraries prepared from the lowest quality sample (duodenum FFPE; 100 ng input only) returned the lowest post-ligation yield, required additional cycles of amplification to achieve the desired final concentration of ≥10 nM, and exhibited a smaller mode fragment size and higher **adapter-dimer rate**, as compared to libraries prepared from higher-quality samples (Table 1). Comparison of adapter-dimer rates for 25 ng vs 100 ng libraries produced from "medium-quality"

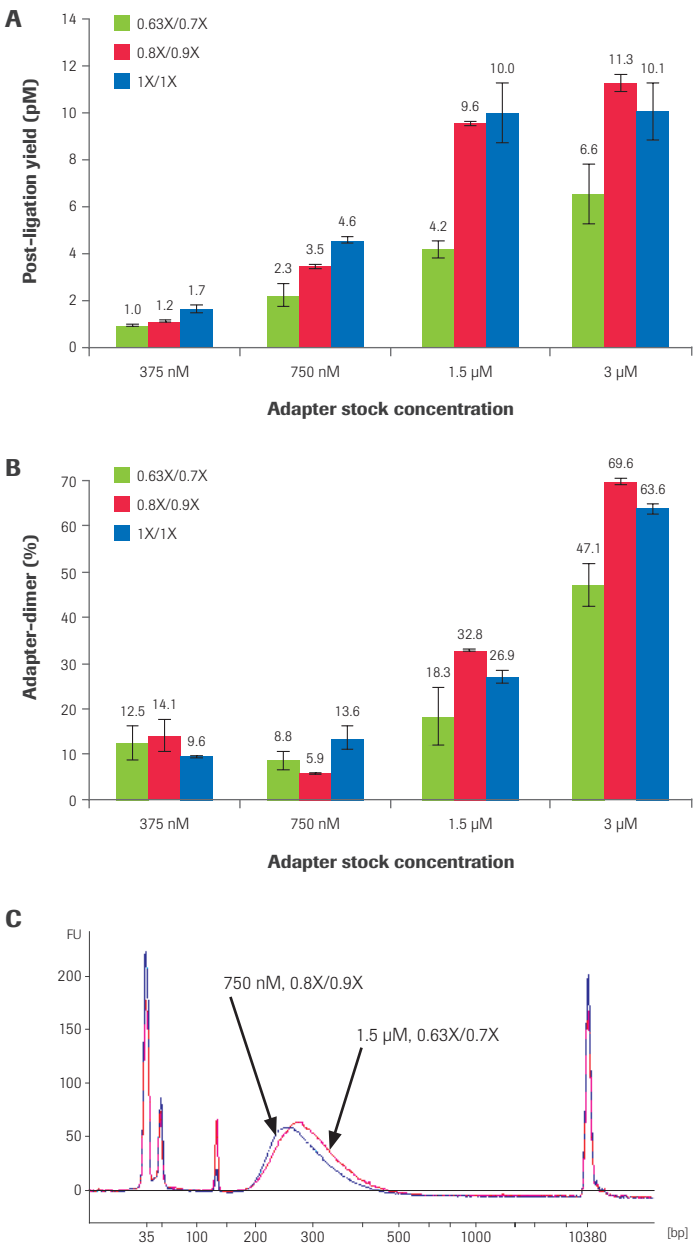


Figure 5: Titration of adapter concentration and post-ligation cleanup ratios. Libraries were prepared from 50 ng inputs of a low-quality RNA extract (DV_{200} = 29%). Post-ligation yields **(A)** were measured by qPCR, whereas adapter-dimer rates **(B)** were calculated from the electrophoretic assessment of final libraries. Each bar represents the mean of two replicate libraries prepared with the KAPA RNA HyperPrep Kit with RiboErase (HMR). **(C)** Electrophoretic analysis confirmed that decreasing the adapter concentration to 750 nM and relaxing post-ligation clean-up ratios to 0.8X, 0.9X lowers adapter-dimer rates without a significant impact on final library yield or size distribution. Standard conditions are an adapter concentration of 1.5 µM, and ratios of 0.63X, 0.7X for the consecutive post-ligation cleanups.

Table 1: Samples, workflow details and select library and sequencing QC data

		UHR		Breast tumor FF		Breast tumor FFPE		Thyroid FFPE		Duodenum FFPE
Input RNA	RIN	8.7		3.0		2.2		2.2		2.5
	DV_{200} (%)	**95**		**74**		**~76 (inflated)**		**47**		**29**
	Input (ng)	25	100	25	100	25	100	25	100	100
Library construction conditions	Fragmentation	94°C for 4 min				65°C for 1 min				
	PCR cycles	13	11	15	13	15	13	15	13	17
Library construction QC data	Post-ligation yield (pM)	9.3	77.5	5.9	17.2	2.4	10.3	5.8	16.3	1.4
	Final library yield (nM)	14.3	22.9	36.1	20.9	8.4	13.6	21.0	26.9	13.9
	Mode library size (bp)	333	349	302	295	278	287	312	305	282
Sequencing metrics	Adapter-dimer (%)	<1.0	<1.0	1.7	<1.0	3.7	3.7	2.6	<1.0	11.7
	rRNA reads (%)	1.6	0.9	4.0	1.5	0.6	0.7	0.4	0.2	1.7

Data on file.

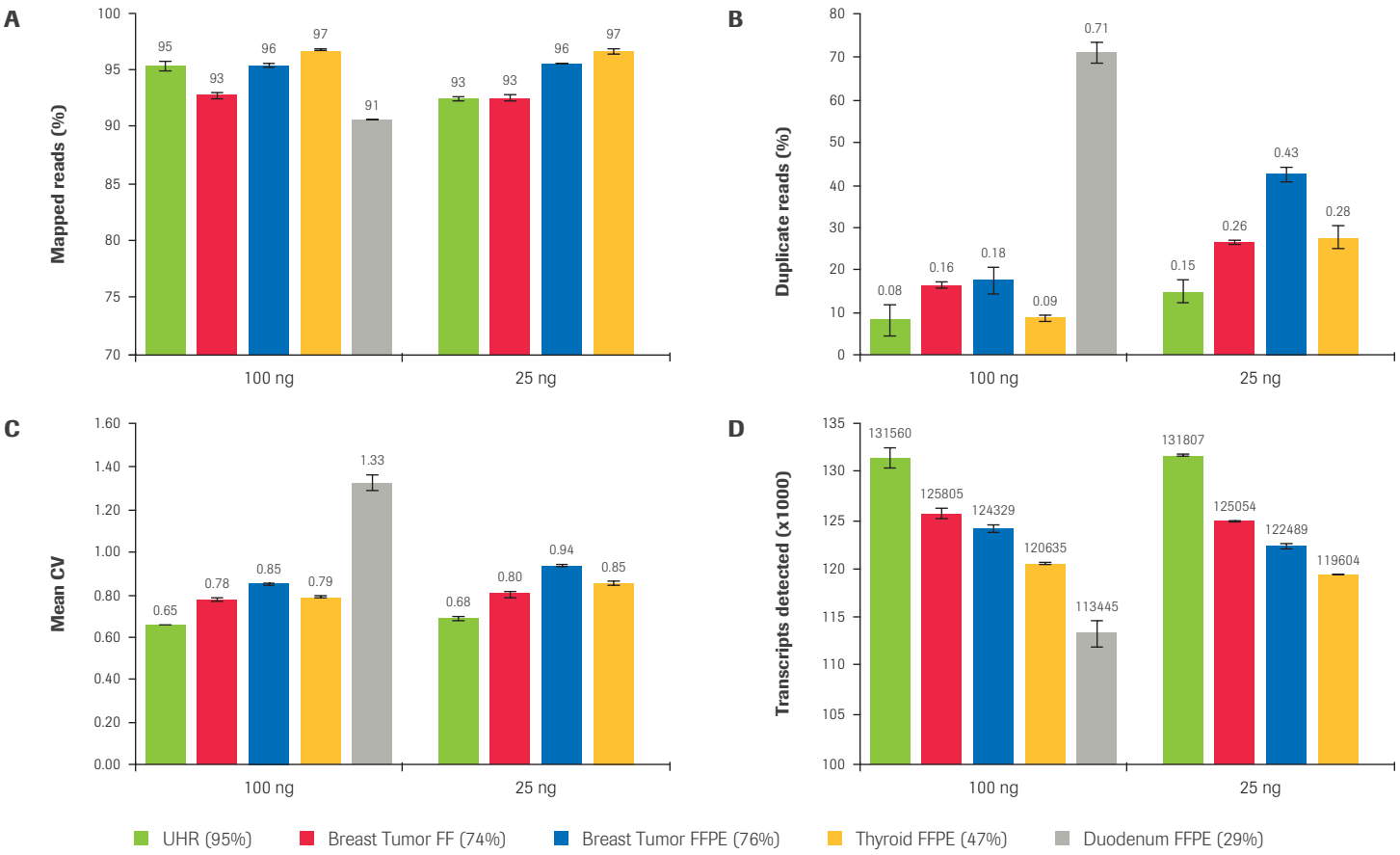


Figure 6: Sequencing QC metrics for libraries prepared with the KAPA RNA HyperPrep Kit with RiboErase (HMR) from RNA samples of variable quality. The DV_{200} value for each sample is given in parentheses after the sample name. Input amounts are given on the x-axis. After subsampling to 14 million reads per sample, data was analyzed to quantify and compare the **(A)** percent mapped reads, **(B)** percent duplicate reads, **(C)** mean CV, and **(D)** number of unique transcripts identified. All bars represent the average of three technical replicates.

thyroid FFPE and breast tumor FFPE and FF samples indicated that library quality can be improved by increasing the input into library construction. When possible, this may be preferred over optimizing adapter concentrations and/or cleanup ratios.

Regardless of RNA quality or input, all samples exhibited **efficient depletion of rRNA**, with less than 5% (<1.3% on average) residual rRNA reads across all libraries. This confirmed that the RNase H-based KAPA RiboErase (HMR) technology offers a robust depletion solution for both high- and low-quality RNA samples.

As shown in Figure 6A, **mapping rates** exceeded 90% for all samples. This demonstrated that the KAPA RNA HyperPrep Kit is suitable for the production of high-quality libraries, regardless of sample quality or input.

Consistent with post-ligation library yields and the number of cycles required to achieve a final library concentration of ≥10 nM, **duplication rates** (Figure 6B) were clearly impacted by sample quality, with the lowest-quality duodenum FFPE sample performing significantly worse than the other libraries prepared from the same input amount (100 ng). For most samples (UHR, breast tumor FFPE and thyroid FFPE), increasing the input into library construction 4-fold (from 25 ng to 100 ng) reduced the required number of amplification cycles proportionately (by 2 cycles), and resulted in an average 3.1-fold reduction in duplication rates.

Coverage uniformity was assessed in terms of mean coefficient of variation (CV) in base coverage across transcript length (Figure 6C). A smaller number reflects less variation in base coverage, or better coverage uniformity. Lower-quality samples exhibited a higher mean CV (lower coverage uniformity) compared to UHR. Increasing input quantity only marginally improved this metric.

Finally, the number of **unique transcripts identified** for each sample was compared (Figure 6D). While there appeared to be a strong correlation between sample quality and transcripts detected, it is important to note that biological factors may have obfuscated this result. RNA was extracted from a variety of tissue types that were not specifically matched in terms of age, metabolic status or environmental stimuli; all of which could have an impact on the expected (maximum possible) number of transcripts for each particular sample. Figure 6D further indicates that input amount had no significant impact on the number of unique transcripts identified. This suggests the amount of sequencing data used in the analysis (14 million reads per sample) was not a limiting factor in the amount of information obtained from each sample.

KAPA RNA HyperPrep Kits with RiboErase (HMR) support reproducible and reliable whole transcriptome sequencing from degraded inputs

Finally, the impact of sample quality on the reliability of sequencing results was assessed. This was done by generating Pearson correlation plots of the TPM values (transcripts per kilobase million;

a measure of gene expression) for replicate libraries produced from the same sample and input amount (Figure 7A – 7C), libraries produced from different inputs of the same RNA extract (Figure 7D), and libraries prepared from matched FF and FFPE tissue samples from the same specimen (Figure 7E and 7F).

Extremely strong correlations (R^2 values ≥0.998) were observed when comparing gene expression profiles for replicate libraries, even for the low-quality duodenum FFPE sample that returned the lowest post-ligation yield, the highest adapter-dimer and duplication rates, and the lowest number of unique transcripts. A similarly high level of reproducibility (R^2 = 0.995) was observed when comparing gene expression data for libraries prepared from different inputs (25 ng vs 100 ng) of the same sample (thyroid FFPE, which had the second lowest DV_{200} value in the set of samples used in this experiment).

When comparing the gene expression profiles for libraries prepared from RNA extracted from the matched FF and FFPE breast tumor tissue samples, the agreement was expected to be lower, due to the larger number of variables associated with sample collection and processing. Nevertheless, very high correlations (R^2 = 0.923 for 100 ng input libraries and R^2 = 0.885 for 25 ng input libraries) were still observed.

Together, these data confirmed that the KAPA RNA HyperPrep Kit with RiboErase (HMR) is highly suitable for the construction of libraries from FFPE-derived samples of variable quality, and supports reproducible and reliable sequencing results.

Conclusions

In this study, the KAPA RNA HyperPrep Kit with RiboErase (HMR) was used to prepare libraries from FFPE-derived RNA extracts of variable quality, to characterize the potential impact of input quality on library and sequencing metrics. Our observations indicated that, compared to higher quality RNA extracts, low-quality or degraded RNA inputs:

- will yield lower quality libraries, with lower post-ligation yields and higher adapter-dimer rates. These outcomes can be mitigated by using the optimized library construction protocol for low-quality RNA inputs,[4] increasing the input into the depletion reaction (if possible), and/or further fine-tuning the ligation reaction parameters (adapter concentration and post-ligation cleanup ratios).
- will require more cycles of amplification to achieve a desired final library concentration, which will increase the number of sequencing reads associated with PCR duplicates.
- may impact coverage uniformity over transcript length if the sample is highly degraded.
- should not result in inefficient RNA depletion, poor mapping rates or poor reproducibility between technical replicates.

Data generated in this study confirmed that the KAPA RNA HyperPrep Kit with RiboErase (HMR) offers a flexible library preparation solution for reliable whole transcriptome sequencing.

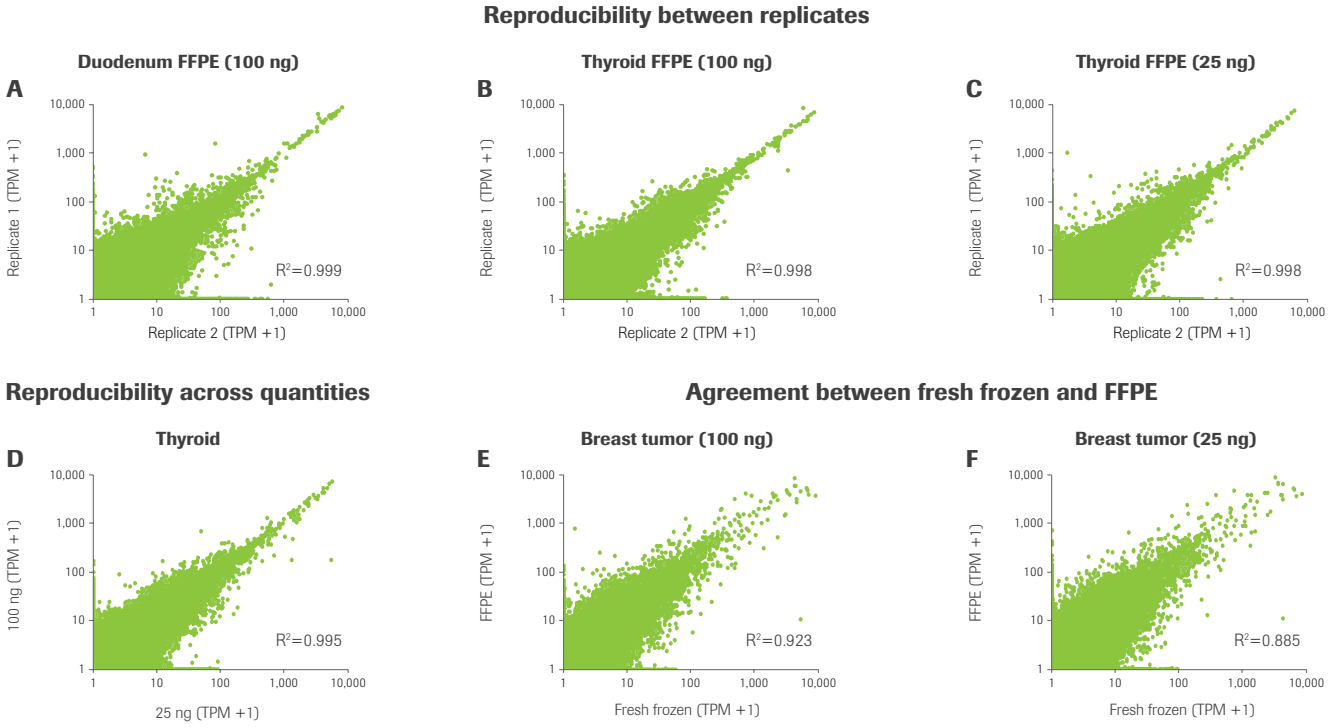


Figure 7: KAPA RNA HyperPrep Kits with RiboErase (HMR) support reproducible and reliable whole transcriptome sequencing from FFPE-derived samples. Pearson correlation plots show extremely high agreement in gene expression data (expressed as transcripts per kilobase million; TPM) for **(A – C)** replicate libraries prepared from the same sample and input, and **(D)** libraries prepared from different input amounts of the same RNA extract. **(E – F)** Gene expression profiles for libraries prepared from matched FF and FFPE breast tumor tissue samples also showed a very high degree of correlation.

In addition to robust performance, the kit offers:

- the convenience of a streamlined, automation-friendly workflow, from input RNA to sequencing-ready library, which can be completed in a standard workday.
- the opportunity to collect QC data at several points in the workflow, to inform key decisions about library construction parameters, and downstream sequencing.
- flexibility to fine-tune key library construction parameters, to improve outcomes for low-quality samples.
- integrated service and support for a complete workflow solution, that includes adapters, and qPCR-based library quantification of final libraries.

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