Application Note

Species-specific ribosomal RNA depletion

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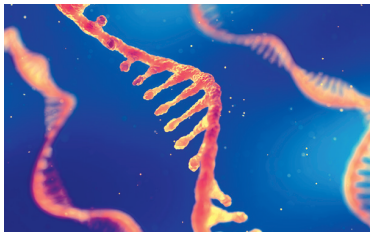
High-efficiency, species-specific ribosomal RNA depletion with the KAPA RNA HyperPrep Kit with RiboErase (HMR)

As ribosomal RNA (rRNA) is extremely abundant, efficient depletion of rRNA is critical to maximize coverage of target RNA and improve sequencing economy in whole-transcriptome next-generation sequencing (RNA-Seq) experiments. The KAPA RiboErase Kit (HMR) is designed for the enzymatic depletion of rRNA from human, mouse and rat samples prior to library construction with the KAPA RNA HyperPrep Kit. The technology is robust and flexible, allowing for the depletion of any known rRNA transcripts with user-designed depletion oligos, including those from organisms other than human, mouse and rat.

Introduction



Ribosomal RNA (rRNA) accounts for up to 80% of total RNA in all cells.[1] Depletion of rRNA is therefore a critical step in generating an enriched transcriptome for downstream applications, and to increase sequencing economy and improve coverage of low-abundance transcripts. In contrast to enrichment methods that capture only poly-adenylated, mature RNA transcripts, selective depletion of rRNA allows for a more comprehensive view of the transcriptome, including precursor mRNAs and long, non-coding (lnc) RNAs. Commercially available kits for RNA depletion utilize either bead-based or enzymatic strategies. The KAPA RiboErase Kit (HMR) employs oligonucleotides complementary to rRNA transcripts and RNase H digestion of DNA:RNA hybrids to enzymatically deplete cytoplasmic and mitochondrial rRNA species from human, mouse and rat samples. This methodology was previously shown to be more effective than bead-based strategies, particularly with low-input samples.[2] The family of KAPA RiboErase Kits was recently expanded to also provide for the depletion of abundant globin mRNA transcripts from human, mouse and rat blood samples.[3]

We have also shown that the KAPA RiboErase protocol can be employed to deplete additional transcripts of choice, using user-designed depletion oligos. Such oligos can simply be added to commercially available rRNA and/or globin oligos, without any downstream protocol modifications.[4]

In this study, we evaluated the utility of the KAPA RiboErase Kit (HMR) for the depletion of rRNA from species other than human, mouse and rat. Bacterial rRNA transcripts were successfully depleted from high-quality *E. coli* RNA, by replacing the HMR rRNA oligos with custom-designed oligos complementary to *E. coli* rRNA transcripts. In parallel, depletion experiments were performed with a bead-based rRNA-depletion kit from a different supplier. The efficiency of rRNA depletion was assessed by qRT-PCR and sequencing on the Illumina® platform. Results indicated that the KAPA RiboErase Kit (HMR) with custom oligos enables more efficient depletion of rRNA from *E. coli* total RNA than the bead-based depletion workflow. This study provides proof-of-concept for the robust and reproducible, species-specific rRNA depletion from any other species.

Experimental design and methods

Oligonucleotide design and preparation

DNA oligonucleotides complementary to *E. coli* 16S rRNA (contained in the 30S ribosomal subunit), as well as 5S and 23S rRNAs (contained in 50S subunit) were designed based on sequences and annotations in the National Center for Biotechnology Information (NCBI) Genbank database. A total of 160 DNA oligos, each ~50 nt in length, were created to span the targeted rRNA sequences. Oligos were purchased from Integrated DNA Technologies (100 µM; standard desalting conditions), and were pooled in equimolar amounts at a final concentration of 1 µg/µL for use in the KAPA RNA HyperPrep Kit with RiboErase workflow. Please **contact Technical Support** for guidelines on the design, ordering and processing of custom oligos, as well as a detailed protocol for setting up the KAPA RiboErase reaction with custom oligos. When first evaluating a new set of species-specific depletion oligos, a titration experiment is recommended to determine the concentration of the oligo pool required for optimal depletion of targeted transcripts.

RNA extraction

MG1655 *E. coli* cells were treated with lysozyme (Sigma) to create spheroplasts, after which RNA extraction was performed with the RNeasy® Mini Kit (QIAGEN®). Following DNase treatment, total RNA was quantified using the Invitrogen™ Quant-iT™ RNA Assay Kit (ThermoFisher). RNA quality was assessed using a 2100 Bioanalyzer™ instrument and an Agilent® RNA 6000 Nano Kit (Agilent Technologies).

Ribodepletion workflow and assessment of depletion efficiency

Two ribodepletion strategies were employed in parallel: the KAPA RiboErase Kit (HMR) with custom depletion oligos and a bead-based, bacterial rRNA removal kit from Supplier I. For each workflow, replicate samples were processed, using either 500 ng (n = 3) or 1000 ng (n = 2) of total RNA as input. User-designed oligos were incorporated in the KAPA RiboErase workflow as described in Figure 1. The Supplier I workflow was performed according to manufacturer's instructions.

To assess the efficiency of rRNA depletion, an aliquot of each depleted RNA sample was removed prior to library construction. A control sample was also taken from the (undepleted) *E. coli* total RNA extract. qRT-PCR was performed using the KAPA SYBR® FAST One-Step qRT-PCR Kit, with primers targeting the *E. coli* 16S rRNA and *cysG* transcripts (Table 1). In this assay, the difference between the calculated Cq value for the 16S rRNA transcript in the depleted vs. control (undepleted) sample (ΔCq) correlates to depletion efficiency (a larger ΔCq indicates more efficient depletion). The *cysG* housekeeping gene was included as an assay control, and for detecting gross instances of off-target depletion. The assay can easily be adopted for any set of targeted transcripts from any species, using an appropriate set of target and control primers, and is highly recommended for the development and optimization of custom depletion workflows.

Table 1. Primers used to assess efficiency of rRNA depletion

Primer Name	Sequence
16S-F	5'-TCC GAA TGG GGA AAC CCA GTG T-3'
16S-R	5'-GGT TCG CCT CAT TAA CCT ATG-3'
cysG-F	5'-GCG CTC GCT TAA CGG TGA AT-3'
cysG-R	5'-CCC TTC GAC GAG GGT TAA CA-3'

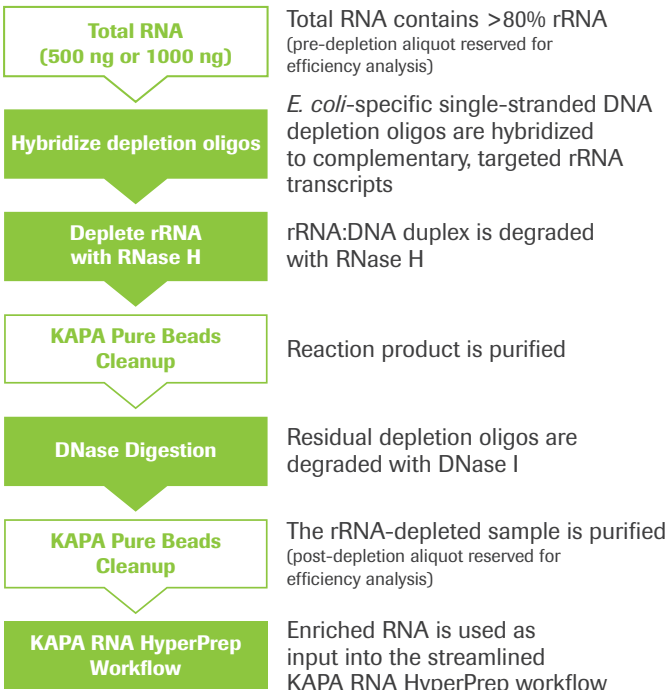


Figure 1. KAPA RiboErase workflow. When setting up the hybridization reaction, the equimolar pool of custom oligos, diluted to a final concentration of 1 µg/µL, was used **instead** of the human, mouse and rat rRNA Hybridization Oligos included in the KAPA RiboErase Kit (HMR). Beyond this substitution, no changes were made to the protocol outlined in the KAPA RiboErase Kit (HMR) Technical Data Sheet.

Library preparation and QC

To assess the impact of the *depletion technology* on the outcome of sequencing, the one library preparation method was used to generate libraries for Illumina sequencing from all depleted RNA samples. The KAPA RNA HyperPrep Kit (Roche), which includes KAPA Pure Beads for reaction cleanups and KAPA HiFi HotStart ReadyMix for library amplification, was used for this purpose; according to instructions outlined in the Technical Data Sheet. Sequencing-ready libraries were quantified using the KAPA Library Quantification Kit (Roche), and size distributions confirmed with 2100 Bioanalyzer instrument and Agilent DNA 7500 Kit (Agilent Technologies).

Sequencing and data analysis

Uniquely-indexed libraries were normalized and pooled. Paired-end (2 x 150 bp) sequencing was performed on an Illumina NextSeq™ instrument, to a high depth (>50 million high-quality reads per sample). Raw sequence files were demultiplexed using bcl2fastq. Low-quality reads were trimmed using Trimmomatic and sequencing read quality was assessed using FastQC. rRNA reads were identified and quantified using SortMeRNA and additional mapping was carried out using Sailfish. RNA-Seq quality metrics were determined by using RNA-SeQC. Differential gene expression data was analyzed using DESeq2. All analysis tools used with default parameters.

Results and discussion

All libraries prepared with the two RNA depletion methods (enzymatic depletion from Roche and bead-based depletion from Supplier I), but the same downstream library preparation protocol (KAPA RNA HyperPrep Kit, Roche) displayed the desired fragment size distribution (Figure 2). The mode fragment size of ~300 bp was consistent with the RNA fragmentation parameters used (94ºC for 6 min).

Results of the qRT-PCR assay used to assess rRNA depletion efficiency are given in Figure 3A. The average ΔCq value (depleted vs. undepleted control) obtained using the 16S primers was almost four cycles higher (11.96 vs. 8.20) for the RiboErase workflow, confirming that the enzymatic RiboErase method is more efficient than the bead-based protocol. As expected, no significant difference in the levels of *cysG* rRNA was observed for either workflow between depleted and undepleted samples (data not shown).

The percentage reads corresponding to residual rRNA, calculated from sequencing data is given in Figure 3B. Data is shown for individual replicate libraries prepared with each of the depletion workflows. This data confirmed the RiboErase workflow to be more efficient, as well as more consistent between replicates.

Other sequencing quality metrics (e.g., mapping, duplication and exonic/intronic rates), were similar for the two depletion workflows (data not shown). This was expected due to the fact that a common library preparation workflow was used downstream of depletion.

Off-target depletion is a common concern in whole-transcriptome sequencing experiments. To assess the risk of off-target depletion with the custom RiboErase workflow, overall gene expression levels for libraries prepared with the two depletion kits were compared. Figure 4 on the next page shows the $\log_2$ fold change distribution of genes found to be differentially expressed between the RiboErase and Supplier I depletion workflows. Highly similar gene expression frequencies, with the $\log_2$ fold change values clustering close to zero suggested that the use of customized, user-supplied oligos does not increase the risk of off-target depletion with the KAPA RiboErase Kit (HMR).

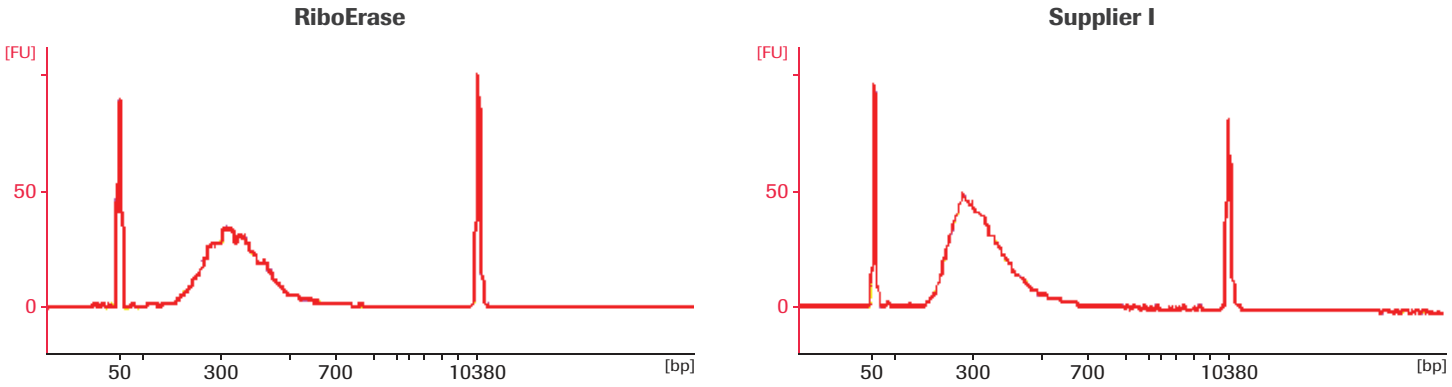


Figure 2. Representative traces of sequencing-ready libraries. Libraries were generated from 500 ng (n = 3) or 1000 ng (n = 2) of *E. coli* total RNA for each depletion strategy. Bacterial rRNA was depleted using either the KAPA RiboErase Kit (HMR) with custom *E. coli* rRNA-specific depletion oligos (left) or a bacterial rRNA removal kit (Supplier I). For all depleted samples, cDNA libraries were subsequently prepared with the KAPA RNA HyperPrep Kit. Electropherograms were generated with a 2100 Bioanalyzer™ instrument and an Agilent® DNA 7500 Kit (Agilent Technologies).

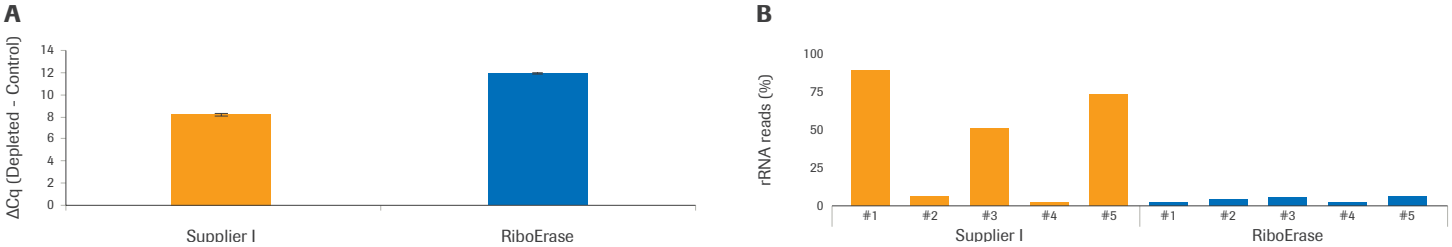


Figure 3. Depletion of *E. coli* rRNA using the KAPA RiboErase Kit (HMR) with custom oligos (orange) is more efficient and consistent than rRNA depletion with a bead-based depletion kit (orange). (A) Results from the qRT-PCR assay with primers targeting the *E. coli* 16S rRNA transcript. The ΔCq value (depleted vs. undepleted control) for the RiboErase workflow was almost four cycles higher than the same value for the Supplier I workflow. **(B)** Percentage of residual rRNA reads calculated from sequencing data, for the five individual libraries prepared with each depletion workflow. For each workflow, libraries 1 – 3 were prepared from 500 ng inputs (into the depletion workflow), and libraries 4 – 5 from 1000 ng inputs.

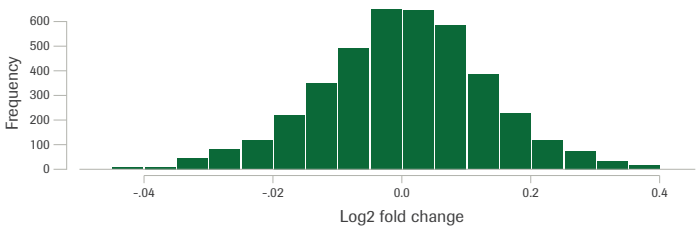


Figure 4. Differential gene expression analysis for the two depletion workflows. Log_2 fold change distribution of differential expression values for libraries prepared using the enzymatic RiboErase vs. bead-based Supplier I workflows. Values cluster around a frequency of 0.0, indicating that the use of custom depletion oligos does not increase the risk of off-target depletion with the RiboErase workflow.

- the ability to collect QC data at several points in the workflow, to inform key decisions about library construction parameters, and downstream sequencing.
- the 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 contains all of the reagents needed for RNA-Seq library construction, as well as qPCR-based library quantification of final libraries.

Conclusions

In this Application Note, we provide evidence that the utility of the KAPA RiboErase Kit (HMR) can be expanded beyond the depletion of rRNA and globin transcripts from human, mouse and rat total RNA extracts. Using custom-designed depletion oligos instead of those provided in commercially available kits, we have shown highly efficient, reproducible and specific depletion of *E. coli* rRNA transcripts from different inputs of total bacterial RNA. We have also confirmed previous findings[2] that RNase H-based depletion protocols outperform bead-based methods.

The results of this, as well as another proof-of-concept study[4], collectively demonstrates the robustness and flexibility of the KAPA RiboErase technology to deplete any RNA species for which the sequences are available. In addition to **replacing** HMR depletion oligos with custom oligo sets (as was done in this study), it was also shown that multiple sets of depletion oligos can be combined in a single experiment. As such, user-designed oligo sets could be used as “spike-ins” to deplete transcripts of microbes or other species from human RNA extracts.

In addition to offering highly efficient depletion of transcripts of choice to improve the sensitivity and sequencing economy of whole-transcriptome sequencing, KAPA RNA HyperPrep Kits with RiboErase (HMR) offer:

- the convenience of a streamlined, automation-friendly workflow, from input RNA to sequencing-ready library, which can be completed in a standard workday.[3]

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