Enhancing the sensitivity and power of transcript detection across species: A customizable, selective, and precise approach for the removal of abundant RNA species

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Introduction

RNA-seq technology has been pushed to extremes of very low and degraded sample inputs but still battles with the challenge of having a large dynamic range of transcript expression. Highly expressed transcripts with minimal biological interest can dominate readouts, masking detection of more informative lower abundant transcripts. Here, we present a customizable approach to enrich for RNAs of interest by eliminating highly abundant and unwanted RNAs. This method is based on hybridization of probes to targeted RNA and subsequent enzymatic degradation of the selected RNAs.
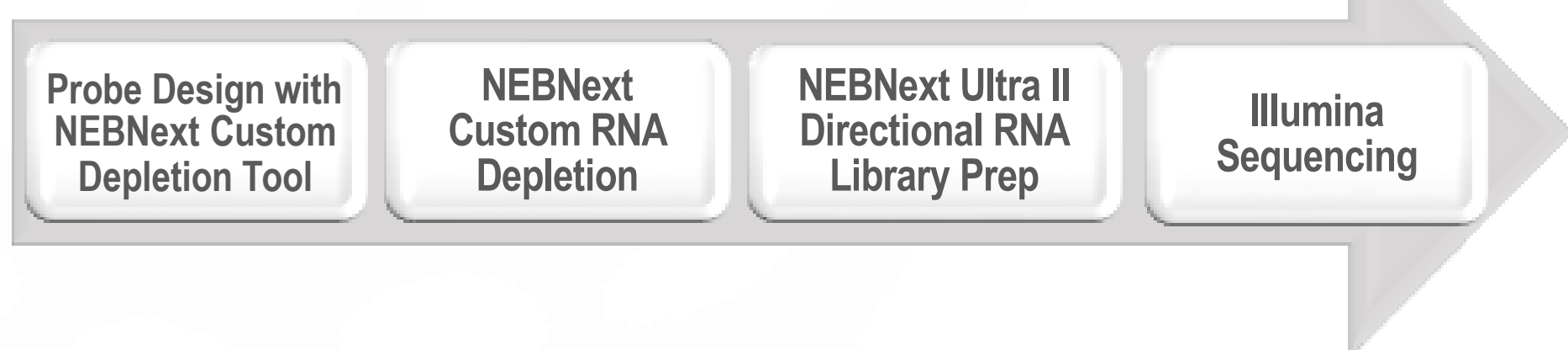
We developed a user-friendly web tool to enable custom probe design for targeted RNA depletion. The custom probe sequences confer RNA removal specificity and can be designed to deplete unwanted RNA from any organism. Furthermore, these probes can be designed to target RNA in single species, multiple species, or to supplement existing probe sets. We used this web tool and depletion method to remove rRNA from total RNA of various species, such as mosquitoes (*Aedes aegypti*) and archaebacteria (*Thermococcus kodakarensis* and *Pyrococcus furiosus*). Additionally, we used this approach to target highly abundant coding RNAs in human total RNA, as a supplement to an existing anti-rRNA probe set, to achieve depletion of both rRNA and the selected coding RNAs.

Using strand-specific RNA sequencing we measured depletion efficiency and transcript expression. We achieved high depletion efficiency (up to 99%) for all targeted RNAs across species, while maintaining transcript abundance of non-targeted RNA. This translated into an enrichment of RNAs of interest and an increased depth of sequencing coverage.

The method and tool described here are a simple and reliable solution that greatly improves the sensitivity and power of RNA-seq studies. Importantly, the targeted RNA depletion and subsequent library construction method are amenable to high throughput sample preparation.
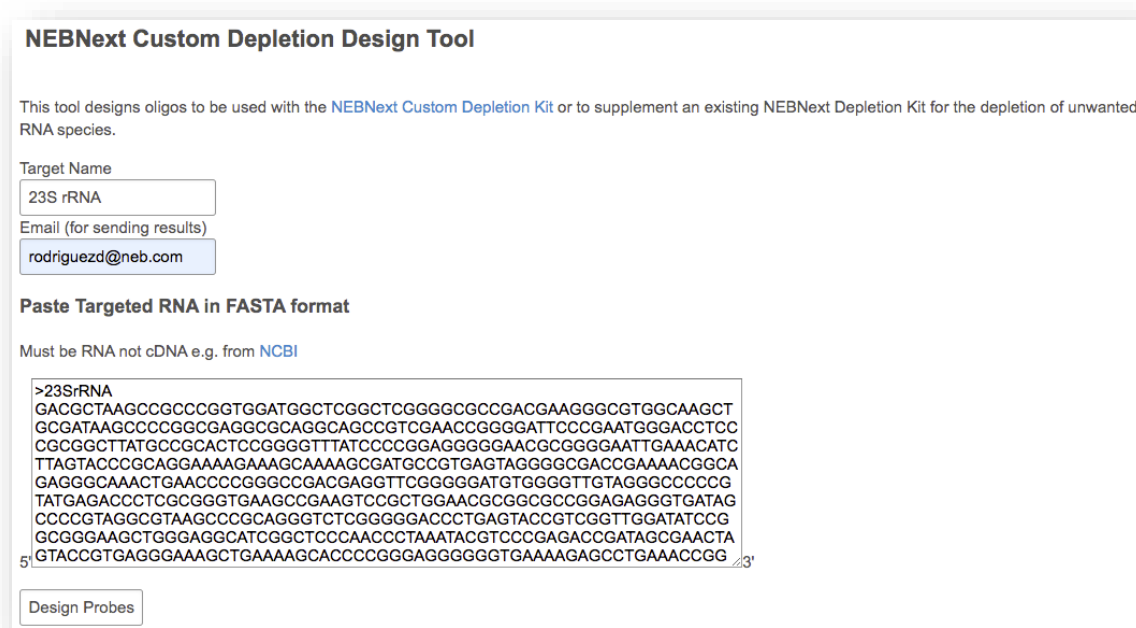
Methods

Experimental Design

Probe Design with NEBNext Custom Depletion Tool → NEBNext Custom RNA Depletion → NEBNext Ultra II Directional RNA Library Prep → Illumina Sequencing
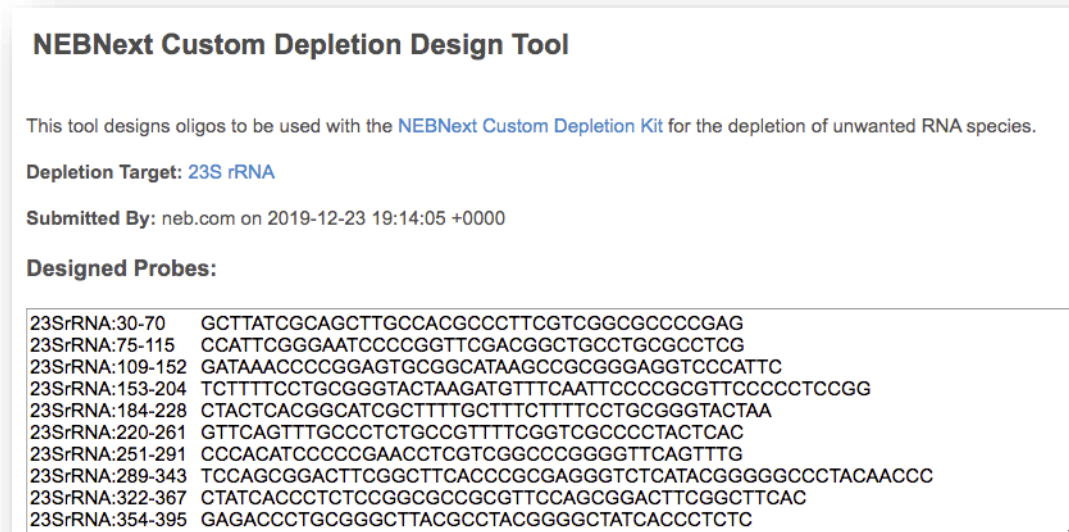
Probe Design with the web-based NEBNext Custom Depletion Tool

Step 1. Enter the sequence of the RNA you want to deplete in fasta format, 5 to 3' orientation. Press the "Design Probes" button

Step 2. The NEBNext web tool outputs probe sequences spanning the input RNA. An email is also sent with the probe sequences
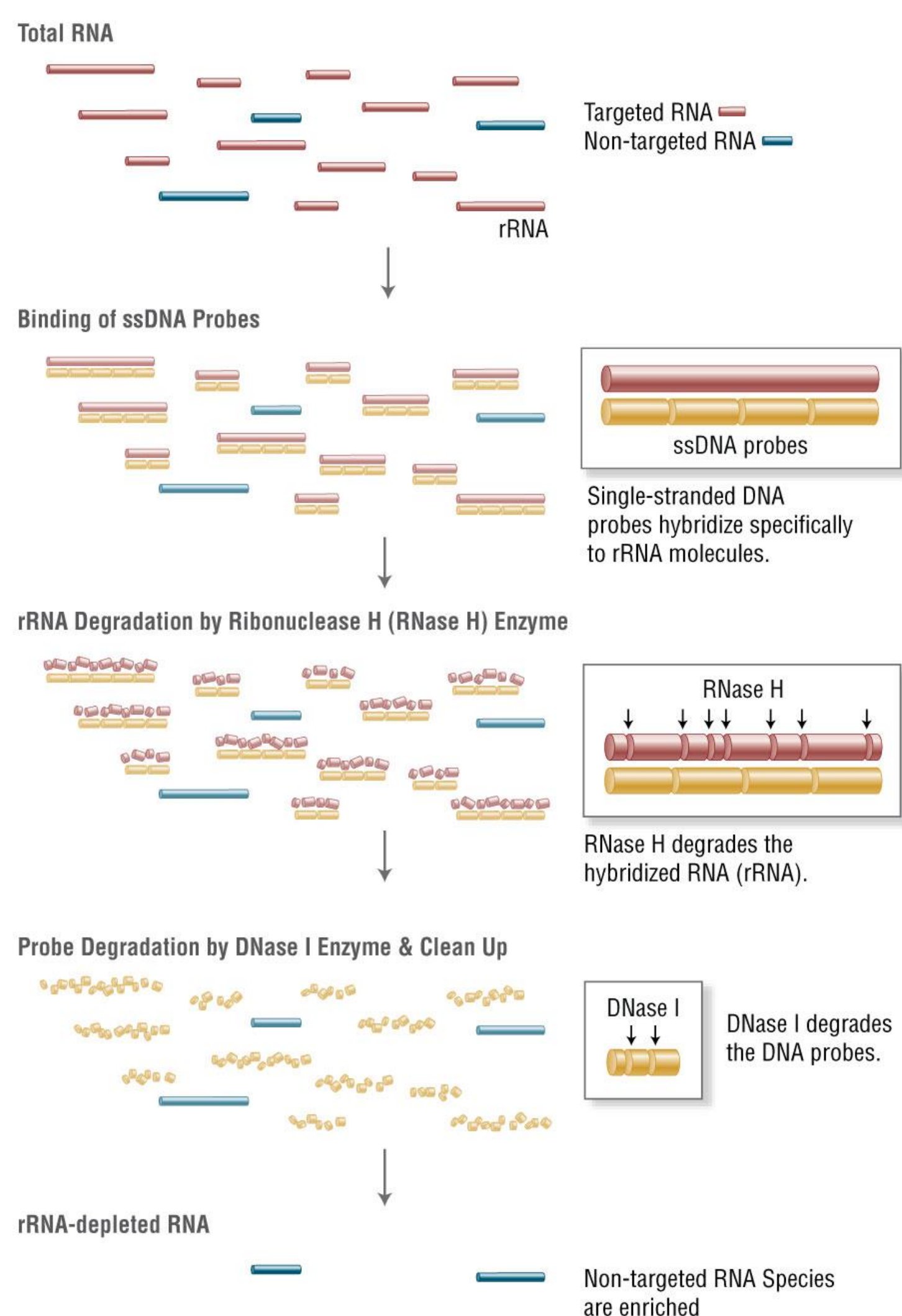
Step 3. Order ssDNA probes from your trusted oligo provider. No modifications needed.

**Step 4.** Use the probes with the NEBNext Custom Depletion Kit

user supplied probes + NEBNext Custom Depletion Kit

Depletion of the Targeted RNA using the NEBNext Depletion Workflow

Total RNA → Targeted RNA / Non-targeted RNA / rRNA

Binding of ssDNA Probes

ssDNA probes — Single-stranded DNA probes hybridize specifically to rRNA molecules.

rRNA Degradation by Ribonuclease H (RNase H) Enzyme

RNase H — RNase H degrades the hybridized RNA (rRNA).

Probe Degradation by DNase I Enzyme & Clean Up

DNase I — DNase I degrades the DNA probes.

rRNA-depleted RNA — Non-targeted RNA Species are enriched

Total RNA is hybridized with ssDNA probes targeting rRNA, followed by RNase H digestion to degrade targeted RNA. The DNA probes are then digested with DNase I, and the reaction is cleaned using magnetic beads. The entire workflow can be done in <2 hours with only 8 minutes of hands-on time. Ribosomal RNA depletion can be immediately followed by RNA-seq library preparation, and sequencing on an Illumina instrument.

Results

Figure 1. Efficient depletion of rRNA from *Aedes aegypti,* and maintenance of non-targeted transcript levels
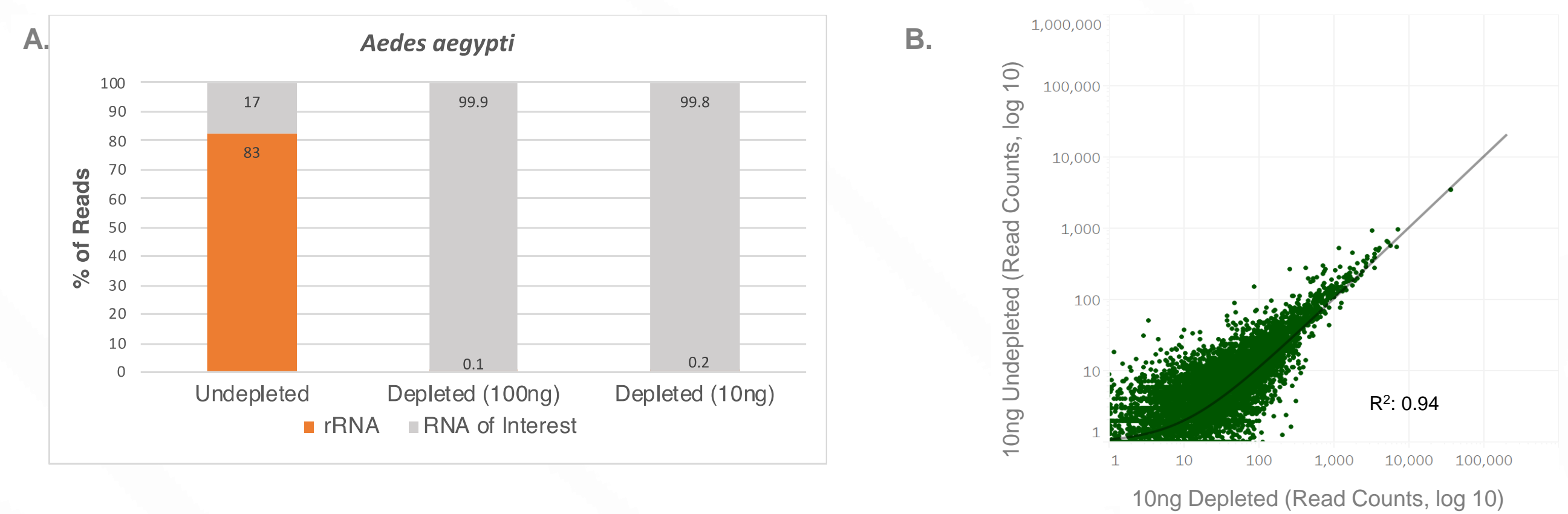
The NEBNext Custom Depletion Design Tool was used to design probes against *Aedes aegypti* rRNA. Adult *Aedes aegypti* mosquitos where purchased from Benzon Research. Total RNA was extracted using the Monarch® Total RNA Miniprep Kit (NEB #T2010S), and 100ng and 10ng used as input for rRNA depletion using the Custom Depletion Kit with the designed probes. RNA-seq libraries were prepared using the NEBNext Ultra™ II Directional RNA Library Prep Kit for Illumina® (NEB #E7760) followed by paired-end sequencing on a NextSeq® instrument (2 x 75 bp). 20 million reads were sampled (seqtk) from each library.
A. Read pairs were identified as ribosomal using mirabait (6 or more, 25-mers), and levels of rRNA remaining were calculated by dividing matched reads by the total number of reads passing instrument quality filtering. The data represents an average of 3 replicates. rRNA depletion enriches for RNAs of interest.
B. Transcript abundances were estimated using Salmon and transcripts from Vectorbase (AaegL5.2 assembly). Read counts and R^2 values for the linear fit are shown. rRNA Depletion does not affect abundances of non-targeted transcripts.

Figure 2. Efficient depletion of rRNA from Archaea total RNA across a wide range of input amounts
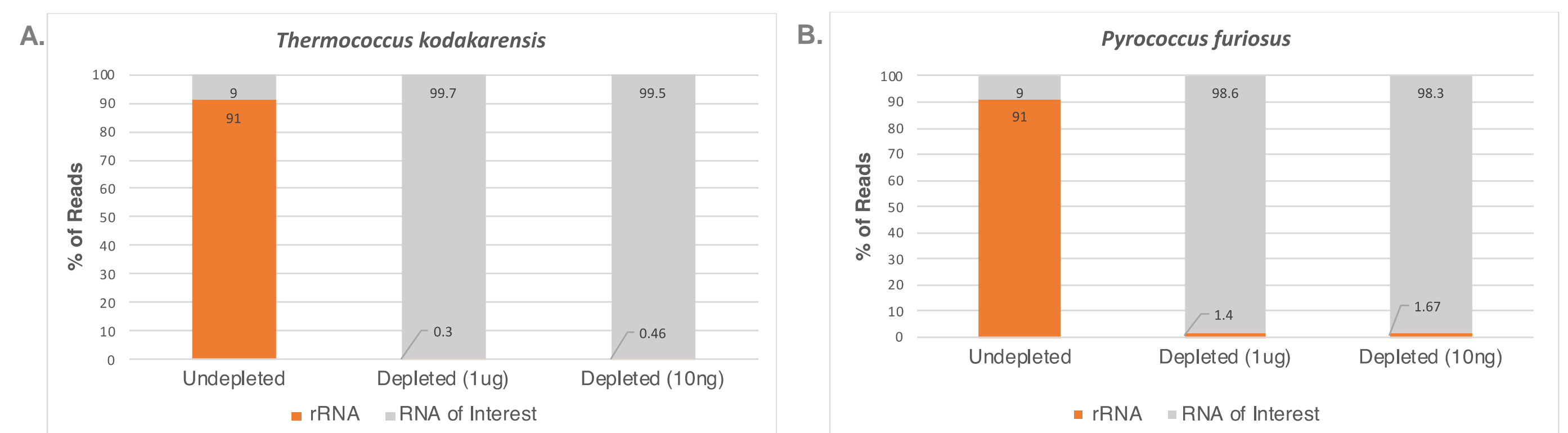
The NEBNext Custom Depletion Design Tool was used to design probes against *Thermococcus kodakarensis* and *Pyrococcus furiosus* rRNA. *Thermococcus kodakarensis* (A) and *Pyrococcus furiosus* (B) total RNA (100ng, 10ng) was depleted of rRNA using the Custom Depletion Kit with the designed probes. RNA-seq libraries were prepared using the NEBNext Ultra™ II Directional RNA Library Prep Kit for Illumina® (NEB #E7760) followed by paired-end sequencing on a NextSeq® Instrument (2 x 75 bp). 20 Million reads were sampled (seqtk) from each library. Read pairs were identified as ribosomal using mirabait (6 or more, 25-mers), and levels of rRNA remaining were calculated by dividing matched reads by the total number of reads passing instrument quality filtering. The data represents an average of 3 replicates. rRNA Depletion greatly enriches for RNAs of interest in both archaea species.

Figure 3. Probe pools are combined to efficiently deplete human rRNA and mitochondrial mRNA
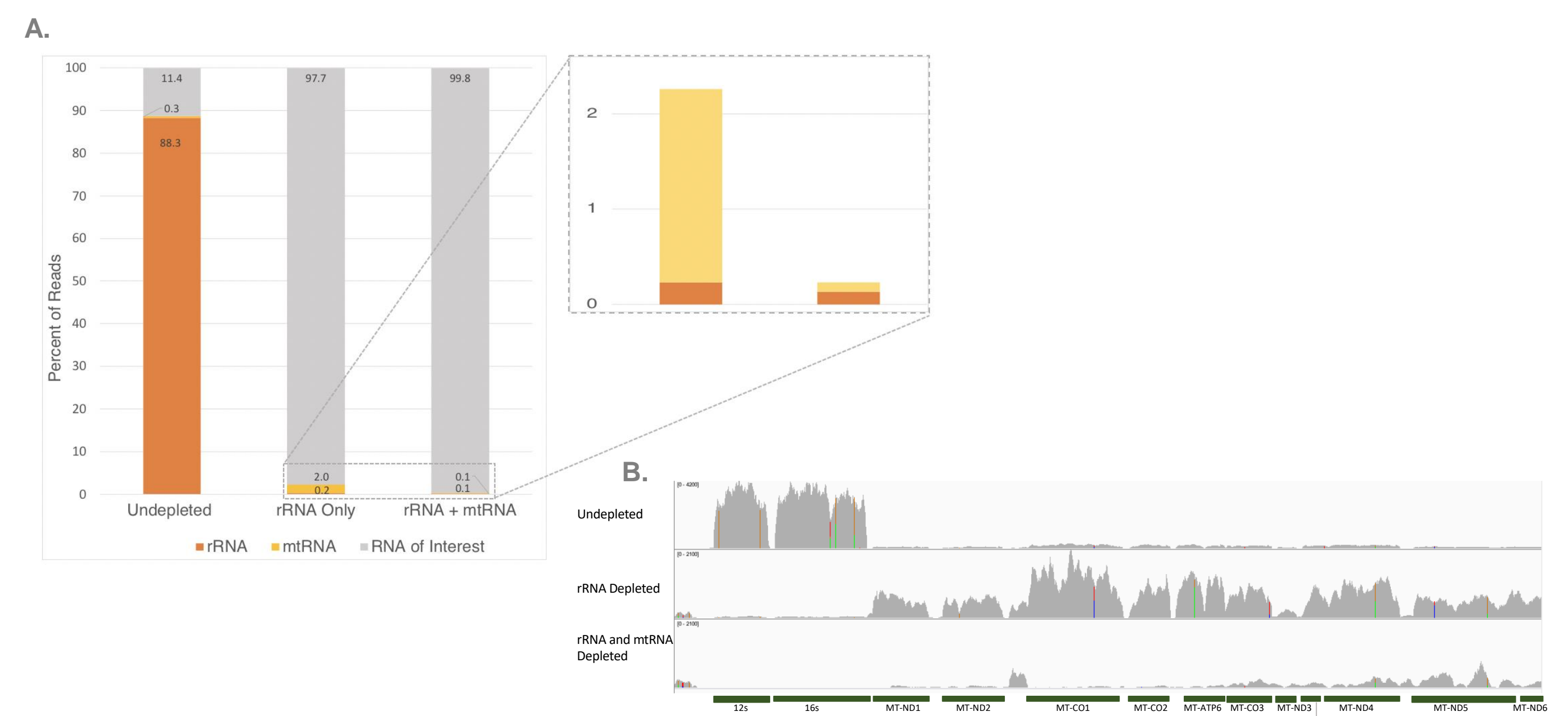
The NEBNext Custom Depletion Design Tool was used to design probes against human mitochondrial mRNA. The probes were used in combination with the NEBNext rRNA Depletion Kit Version 2 (Human/Mouse/Rat) probe pool. Total universal human reference RNA (1ug) was depleted of mitochondrial RNA and rRNA using the Custom Depletion Kit. RNA-seq libraries were prepared using the NEBNext Ultra™ II Directional RNA Library Prep Kit for Illumina® (NEB #E7760) followed by paired-end sequencing on a NextSeq® instrument (2 x 75 bp). 20 Million reads were sampled (seqtk) from each library.
A. Read pairs were identified as ribosomal and mitochondrial using mirabait (6 or more, 25-mers), and levels of rRNA and mtRNA remaining were calculated by dividing matched reads by the total number of reads passing instrument quality filtering. Both rRNA and mitochondrial RNA are efficiently depleted.
B. Integrative Genome Viewer (IGV) visualization of read coverage across the human mitochondrial genes.

Conclusions

- The NEBNext Custom Depletion Tool facilitates the design of probes to remove unwanted RNA in any organism of interest.
- The probes are used in conjunction with the NEBNext Custom Depletion Kit to efficiently remove unwanted RNA.
- The method is amenable for a wide range of inputs (10ng-1ug total RNA).
- Depletion does not affect transcript abundances of RNA species not targeted.
- Both coding and noncoding RNAs are recovered after depletion (if not targeted).
- Designed probes can be combined with existing probe-pools for a more customized experimental setup.
- Successful depletion was achieved using this method on total RNA from *Aedes aegypti*, *Thermococcus kodakarensis*, *Pyrococcus furiosus*, and *Homo sapiens*.
- Depletion of highly abundant transcripts, such as rRNA, greatly increased the number of reads mapping to RNAs of interest.