

Selective Depletion of Abundant RNAs to Enable Transcriptome Analysis of Low-input and Highly-degraded RNA from FFPE Breast Cancer Samples

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Introduction

Deep sequencing of cDNA prepared from total RNA (RNA-seq) has become the method of choice for transcript profiling and discovery. However, the standard whole-transcriptome approach faces a significant challenge, as the vast majority of reads map to ribosomal RNA (rRNA). One solution is to enrich the sample RNA for polyadenylated transcripts using oligo (dT)-based affinity matrices; although, this also eliminates other biologically relevant RNA species, such as microRNAs and noncoding RNAs, and relies on having a high-quality and high-quantity RNA sample. Here, we present a method to eliminate abundant RNAs from total RNA with different degradation levels, from intact RNA to highly degraded formalin-fixed paraffin-embedded (FFPE) samples. This method is based on hybridization of probes to the targeted abundant RNA, followed by subsequent enzymatic degradation (Fig. 1, 2).

 **FIGURE 1:**
NEBNext rRNA Depletion Kit workflow input amounts and times.

Kit	Input Amount	Time				Workflow Time
		RNA/Probe Hybridization	RNase H Digestion	DNase I Digestion	Clean Up	
10 ng – 1 µg	Hands-On	2 min.	2 min.	2 min.	2 min.	8 min.
	Total	22 min.	32 min.	32 min.	27 min.	1 hr., 53 min.

Materials

NEBNext[®] rRNA Depletion Kit (Human/Mouse/Rat) (NEB #E6310)

RNA samples of high and low quality, including FFPE samples

Universal Human Reference RNA (UHR, Agilent[®])

Bioanalyzer[®] with DNA High-sensitivity and RNA Pico Chips

NEBNext Oligos for Illumina (NEB #E7335, E7350, E7500)

NEBNext Ultra[™] Directional RNA Library Prep Kit (NEB #E7420)

Magnetic rack

Thermocycler

Agencourt[®] RNAClean[®] XP (Beckman-Coulter, Inc. #A63987)

Agencourt AMPure DNA XP (Beckman-Coulter, Inc. #A63881)

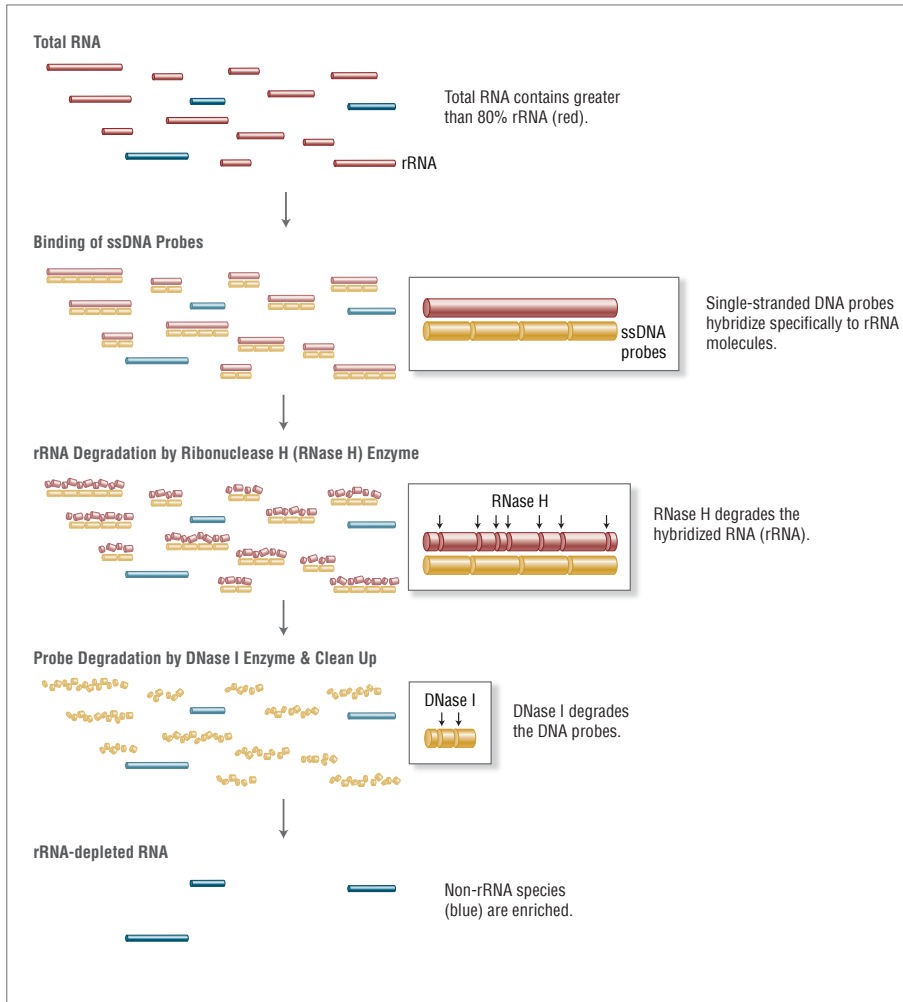
Materials

Depletion of rRNA from Total RNA from FFPE human RNA, as well as high-quality human, mouse and rat RNA

We applied this method to remove cytoplasmic and mitochondrial rRNA from different eukaryotic total RNA samples (human, mouse and rat), as well as degraded (archive age of 1 year) and highly-degraded (archive age of 10 years) human FFPE breast tumor biopsy RNA samples.

FIGURE 2:
NEBNext rRNA Depletion Kit workflow.

Total RNA (0.1-1 µg) is hybridized with single stranded DNA probes targeting cytoplasmic (5S, 18S, 28S, 5.8S rRNAs) and mitochondrial (12S and 16S rRNAs) ribosomal RNA, followed by RNase H digestion to degrade targeted RNA. Finally, DNA probes are digested with DNase I. The ribosomal-depleted RNA is purified using Agencourt RNAClean XP beads. Ribosomal RNA depletion can be immediately followed by RNA-seq library preparation.



General Protocol

Preparation of RNA/Probe Master Mix

1. Prepare the following RNA/Probe master mix, on ice:

COMPONENT	VOLUME
NEBNext rRNA Depletion Solution	1 µl
Probe Hybridization Buffer	2 µl
Total Volume	3 µl

2. Add 3 µl of the above mix to 12 µl of total RNA sample
3. Mix by pipetting up and down.
4. Spin down briefly in a tabletop centrifuge and proceed immediately to the next step.

- Place samples in a thermocycler, and run the following program, which will take approximately 15–20 minutes:

TEMP	TIME
95°C	2 minutes
95-22°C	0.1°C/second
22°C	5 minute hold

- Spin down the samples in a tabletop centrifuge, and place on ice. Proceed immediately to the next step.

RNase H Digestion

- Prepare the following master mix on ice, and use immediately:

COMPONENT	VOLUME
RNase H	2 µl
RNase H Reaction Buffer	2 µl
Nuclease-free Water	1 µl
Total Volume	5 µl

- Mix by pipetting up and down.
- Add 5 µl of the above mix to the RNA sample from Step 6.
- Mix by pipetting up and down.
- Place samples in a thermocycler (with lid at 40°C), and incubate at 37°C for 30 minutes.
- Spin down the samples in a tabletop centrifuge, and place on ice. Proceed immediately to the next step to prevent non-specific degradation of RNA.

DNase I Digestion

- Prepare the following DNase I Digestion Master Mix on ice, and use immediately:

COMPONENT	VOLUME
DNase I Reaction Buffer	5 µl
DNase I (RNase-free)	2.5 µl
Nuclease-free Water	22.5 µl
Total Volume	30 µl

- Mix by pipetting up and down.
- Add 30 µl of the above mix to the RNA sample from Step 12, and mix by pipetting up and down.
- Place samples in a thermocycler (with lid at 40°C), and incubate at 37°C for 30 minutes.
- Spin down the samples in a tabletop centrifuge, and place on ice. Proceed immediately to the next step.

RNA Purification after rRNA Depletion

- Add 110 µl (2.2X) Agencourt RNAClean XP beads to the RNA sample from Step 17, and mix by pipetting up and down.
- Incubate samples on ice for 15 minutes.
- Place the tube on an appropriate magnetic rack to separate the beads from the supernatant.
- When the solution is clear (about 5 minutes), discard the supernatant.
- Add 200 µl of freshly-prepared 80% ethanol to the sample while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.

23. Repeat Step 22 once, for a total of 2 washes.
24. Briefly spin the tube, and put the tube back in the magnetic rack.
25. Completely remove the residual ethanol, and air dry the beads.
26. Remove the tube from the magnetic rack. Elute RNA from the beads with 8 μ l of nuclease-free water.
27. Mix well by pipetting up and down, and put the tube in the magnetic rack, until the solution is clear.
28. Transfer 6 μ l of the supernatant to a clean PCR tube.
29. Place the sample on ice, and proceed with NGS library construction or other downstream application.

Results

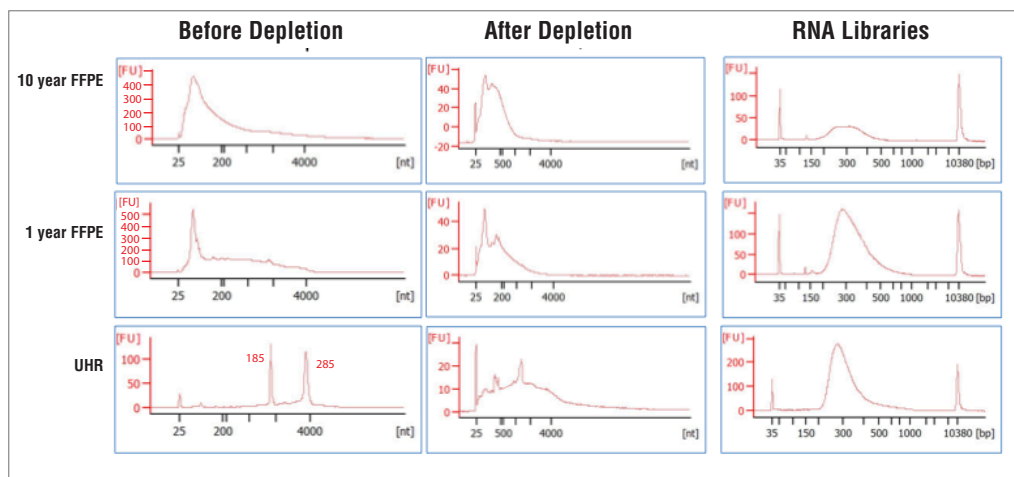
The NEBNext rRNA Depletion Kit is effective on RNAs of varying quality, from low quality (FFPE 1–10 years) to high quality

Multiple RNA samples were processed using the NEBNext rRNA Depletion Kit in order to examine effectiveness with different RNA degradation levels: “10 year FFPE RNA” was a pool of equal portions of RNA extracted from eight FFPE breast cancer samples with an approximate archive age of 10 years. “1 year FFPE RNA” was a pool of equal portions of RNA extracted from > 100 breast tumor biopsy samples with an archive age of one year. “UHR”, or Universal Human Reference RNA, is a commercially available non-degraded RNA (Agilent). RNA profiles before and after rRNA depletion were analyzed by an Agilent Bioanalyzer on an RNA Pico chip (Figure 3).

Directional RNA-Seq libraries were then made from rRNA-depleted RNA and from non-depleted total RNA (not shown) using the NEBNext Ultra Directional RNA Library Prep Kit for Illumina® (NEB #E7420). Libraries were analyzed on a Bioanalyzer using a DNA High sensitivity chip (Figure 3).

 **FIGURE 3: Comparison of rRNA depletion on RNA samples of varying quality.**

RNA samples of varying quality were analyzed on a Bioanalyzer using an RNA Pico chip before and after rRNA depletion, and depleted libraries were analyzed on a Bioanalyzer using a High-Sensitivity DNA chip.



As can be seen in the intact UHR sample, two distinct peaks, corresponding to the ribosomal subunits 18S and 28S, are visible in the sample that was not depleted (“Before Depletion”). These peaks are not visible in the 10 year or 1 year FFPE samples, because the RNA is degraded. After depletion, the 18S and 28S peaks are no longer present, indicating that rRNA depletion was successful. All rRNA-depleted samples were used to make high-quality RNA libraries, with optimal size distribution for subsequent RNA-seq experiments.

The NEBNext rRNA Depletion Kit offers superior performance with fragmented and FFPE RNA

To further examine rRNA depletion efficiency, performance was assessed on different input amounts of FFPE RNA, and on intact and fragmented UHR RNA. Total RNA was treated with the NEBNext rRNA Depletion Kit, Ribo-Zero® Gold (Epicentre®) or the Ribo-Zero Gold rRNA depletion reagents provided within the TruSeq® Stranded Total RNA Kit with Ribo-Zero Gold (Illumina).

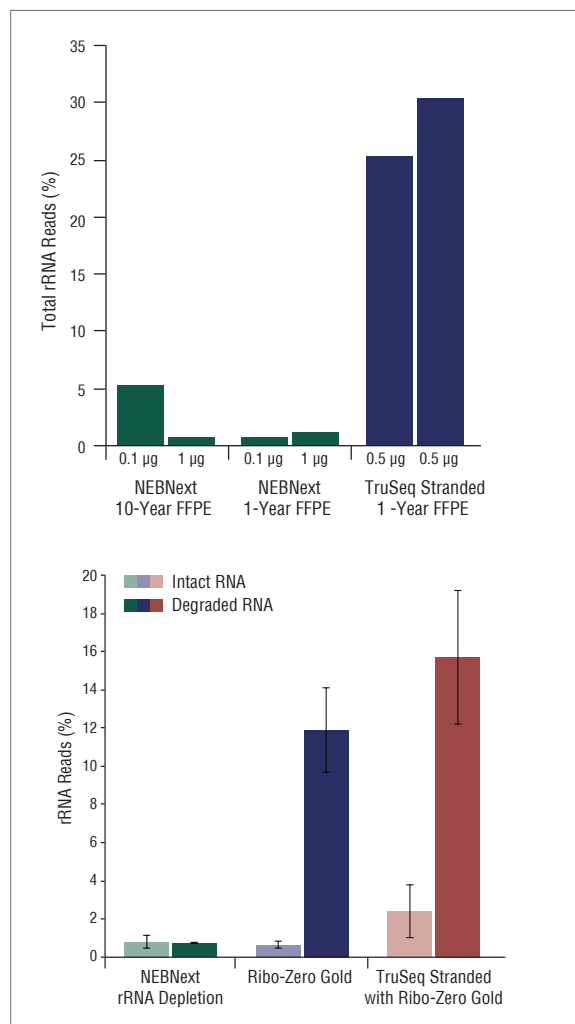
Libraries were made from samples depleted of rRNA and sequenced on the Illumina platform. The percentage of sequence reads that aligned to rRNA was then determined.

NEBNext rRNA-depleted libraries contained a minimal percentage of total reads that aligned to rRNA subunits, regardless of the quality of the input RNA (FFPE, Fragmented or intact UHR). In contrast, Ribo-Zero rRNA-depleted libraries resulted in a higher percentage of reads (> 10% total reads) mapping to rRNA for the degraded samples (FFPE RNA or fragmented RNA).



FIGURE 4:
rRNA depletion efficiency.

rRNA was depleted from human breast cancer FFPE Total RNA (Panel A) or from intact and fragmented Universal Human Reference Total RNA (UHR, Panel B) using the NEBNext rRNA Depletion Kit, Ribo-Zero Gold (Epicentre #MRZG126) or the Ribo-Zero Gold reagents provided within the TruSeq™ Stranded Total RNA Kit (Illumina #RS-122-2201A). rRNA-depleted RNA libraries were made using either the NEBNext Ultra Directional RNA Library prep (NEB #E7420) or the TruSeq Stranded Total RNA Kit (#RS-122-2201A). Total reads were aligned to the cytoplasmic (5S, 18S, 5.8S and 28S) and mitochondrial (12S and 16S) ribosomal RNA subunits using Bowtie 2.0 (local, sensitive).



Transcript Composition

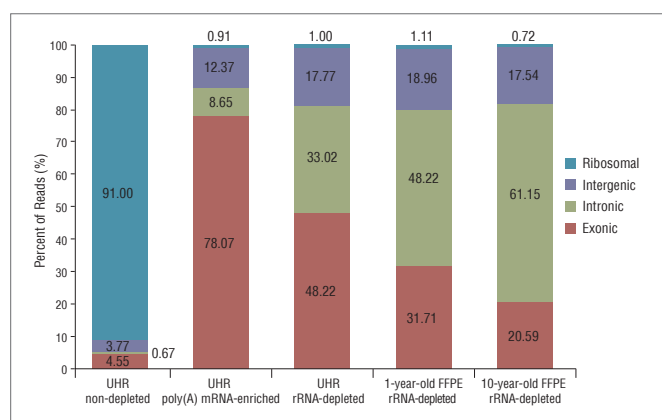
The composition of transcripts after rRNA depletion was assessed by determination of the proportion of reads mapping to annotated exons, introns and intergenic regions, and this was compared to the composition of non-depleted total RNA and of poly(A) mRNA-enriched RNA.

Libraries generated from rRNA-depleted RNA resulted in low rRNA reads, comparable to poly(A) mRNA-enriched RNA, while also retaining more noncoding reads. Effective rRNA depletion efficiency was achieved even with FFPE RNA. The exonic ratio was constant between total RNA input amounts of 100ng (shown) and 1µg (not shown). FFPE RNA contained a higher percentage of intronic reads than fresh RNA, as previously reported (1).



FIGURE 5:
Read distribution across transcripts.

RNA-seq libraries were generated from Universal Human Reference Total RNA (UHR, Agilent) or Breast Cancer FFPE RNA (with an archive age of 1 year and 10 years). RNA was either untreated or treated with the NEBNext poly(A) mRNA Magnetic Isolation Module (NEB #E7490) or the NEBNext rRNA Depletion Kit. RNA-seq libraries were made using the NEBNext Ultra Directional RNA Library Prep Kit for Illumina (NEB #E7420). Reads were mapped to the hg19 genome and read distributions were determined using Picard RNA-Seq Metrics.



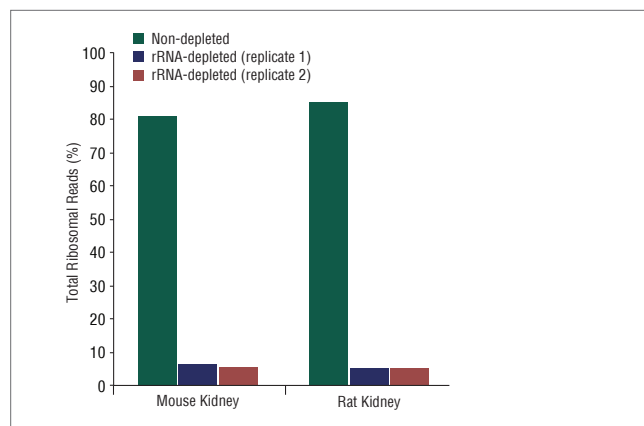
The NEBNext rRNA Depletion Kit also effectively depletes rRNA from mouse and rat samples

To assess rRNA depletion efficiency for other eukaryotic total RNA, rRNA was depleted from mouse and rat kidney total RNA using the NEBNext rRNA depletion method. Libraries were made and sequenced on the Illumina platform, and the corresponding percentage of rRNA-aligned reads was determined.



FIGURE 6
rRNA depletion in mouse and rat.

Ribosomal RNA was depleted from mouse and rat kidney total RNA (two technical replicates) using the NEBNext rRNA Depletion Kit. RNA-seq libraries were made from non-depleted total RNA or rRNA-depleted RNA, using the NEBNext Ultra Directional RNA Library Prep Kit for Illumina (NEB #E7420). Total rRNA-aligned reads were determined using Bowtie 2.0 (local, sensitive). NEBNext rRNA-depleted mouse and rat libraries contain a minimal percentage of reads mapping to rRNA.



Transcript expression correlation with non-depleted sample libraries

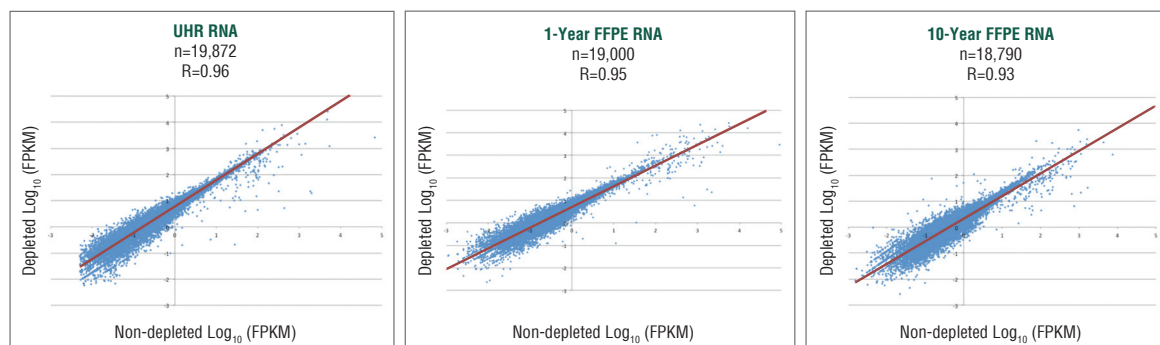
To investigate any effects on non-ribosomal RNA, correlation of transcript expression was determined between rRNA-depleted and non-depleted samples, for UHR and FFPE samples. FPKM (Fragments Per Kilobase of transcript per Million mapped reads) correlation analysis of sequencing reads from libraries generated with these samples indicated very good transcript expression correlation ($R > 0.93$) between rRNA-depleted and non-depleted libraries. Moreover, NEBNext rRNA depletion did not alter transcript expression levels.



FIGURE 7

Transcript expression correlation between depleted and non-depleted libraries.

Libraries were made from UHR RNA (Agilent) and Breast Cancer FFPE RNA (with archive age of one year and 10 years), both non-depleted RNA and RNA depleted using the NEBNext rRNA Depletion Kit. All libraries were made using the NEBNext Ultra Directional RNA Library Prep Kit for Illumina (NEB #E7420). TopHat2 and Cufflinks were used for read mapping and transcript assembly and quantification.



Conclusion

Regardless of the quality or amount of input RNA, this method efficiently removes rRNA, while retaining non-coding and other non-poly(A) RNAs that are lost with oligo d(T) poly(A) mRNA enrichment methods, offering a more complete picture of the transcript repertoire.

Reference:

1. Morlan, J.D., *et al.* (2012) Selective depletion of rRNA enables whole transcriptome profiling of archival fixed tissue. *PLOS One* 7:e42882.

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