



## A reverse transcriptase-mediated ribosomal RNA depletion (RTR2D) strategy for the cost-effective construction of RNA sequencing libraries

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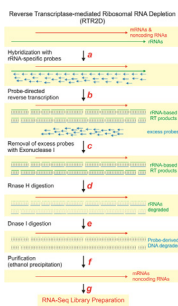
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### GRAPHICAL ABSTRACT



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### ABSTRACT

RNA sequencing (RNA-seq)-based whole transcriptome analysis (WTA) using ever-evolving next-generation sequencing technologies has become a primary tool for coding and/or noncoding transcriptome profiling. As WTA requires RNA-seq data for both coding and noncoding RNAs, one key step for obtaining high-quality RNA-seq data is to remove ribosomal RNAs, which can be accomplished by using

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various commercial kits. Nonetheless, an ideal rRNA removal method should be efficient, user-friendly and cost-effective so it can be adapted for homemade RNA-seq library construction. Here, we developed a novel reverse transcriptase-mediated ribosomal RNA depletion (RTR2D) method. We demonstrated that RTR2D was simple and efficient, and depleted human or mouse rRNAs with high specificity without affecting coding and noncoding transcripts. RNA-seq data analysis indicated that RTR2D yielded highly correlative transcriptome landscape with that of NEBNext rRNA Depletion Kit at both mRNA and lncRNA levels. In a proof-of-principle study, we found that RNA-seq dataset from RTR2D-depleted rRNA samples identified more differentially expressed mRNAs and lncRNAs regulated by Nutlin3A in human osteosarcoma cells than that from NEBNext rRNA Depletion samples, suggesting that RTR2D may have lower off-target depletion of non-rRNA transcripts. Collectively, our results have demonstrated that the RTR2D methodology should be a valuable tool for rRNA depletion.

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**Introduction**

RNA sequencing (RNA-seq)-based whole transcriptome analysis (WTA) has increasingly become a primary tool for the coding and/or noncoding transcriptome profiling in order to decipher genome function, identify genetic networks underlying physiological and biochemical systems, and investigate RNA biology, as well as to characterize potential biomarkers that predict or diagnose diseases, pathogens and environmental challenges [1,2]. The ever-evolving next-generation sequencing technologies have already enabled us to analyze single-cell gene expression, translation (the translome), RNA structure (the structurome), and/or spatial transcriptomics (spatialomics) [1,2]. Furthermore, while the Illumina short-read sequencing technology has accounted for >95% of the published RNA-seq data available on the Short Read Archive (SRA), the long-read cDNA sequencing and direct RNA-seq technologies ushered by Pacific Biosciences (PacBio) and Oxford Nanopore (ONT) may soon provide a fuller understanding of RNA biology, including differential isoform expression, base modification detections, and the folding and intermolecular interactions that govern RNA function [1].

Regardless of the methods, the standard workflow of RNA-seq library preparation begins with total RNA isolated from samples/cells of interest. Historically, RNA-seq was developed to analyze polyadenylated transcripts (e.g., mRNA), and the majority of published RNA-seq data have been generated from oligo-dT-enriched mRNA, which only focuses sequencing on the protein-coding regions of the transcriptome. However, such method is inherited flawed as it is 3'-end biased and fails to capture noncoding RNAs, such as microRNAs, enhancer RNAs, and many long noncoding RNAs (lncRNAs) [1]. Thus, whole transcriptome analysis (WTA) requires the production of RNA-seq data from both coding and noncoding RNAs. One key step for obtaining high quality RNA-seq data, especially for short-read cDNA sequencing, is to completely remove ribosomal RNAs, which otherwise may account for up to 95% of total reads [3]. For many WTA studies, other high abundance transcripts, such as mitochondrial rRNAs (12S and 16S rRNAs) or globin RNAs, also need to be depleted.

Ribosomal RNA (rRNA) production represents the most active transcription in the cell and account for approximately 80% of total RNAs [4,5]. The mature 28S, 18S and 5.8S rRNAs in higher eukaryotes are encoded by a single pre-rRNA transcription unit, which is simultaneously transcribed by numerous RNA polymerase I enzymes as a 45S primary transcript (pre-rRNA) and processed into mature rRNAs found in cytoplasmic ribosomes [4,5]. Currently, rRNA removal can be accomplished in two general approaches, by separating rRNAs from other RNA transcripts using biotinylated probes and streptavidin-coated magnetic beads (or so-called pull-out), or by selective degradation of rRNA by RNase H [1]. These approaches employ sequence- and species-specific oligonucleotide probes that are complementary to both cytoplasmic 5S rRNA, 5.8S

rRNA, 18S rRNA and 28S rRNA and mitochondrial 12S rRNA and 16S rRNA [1].

Commercially available pull-out kits include Ribo-Zero (Illumina, USA) and RiboMinus (Thermo Fisher, USA), while the RNase H-based rRNA degradation of oligo-DNA:RNA hybrids includes RiboErase (Kapa Biosystems, USA) and NEBNext rRNA Depletion (New England Biolabs, NEB), which depletes both cytoplasmic (5S rRNA, 5.8S rRNA, 18S rRNA and 28S rRNA) and mitochondrial ribosomal RNA (12S rRNA and 16S rRNA) from human, mouse and rat total RNA preparations. While both approaches may be able to reduce rRNAs to < 20% of the subsequent RNA-seq reads, rRNA depletion approaches generally require a higher read depth per sample than oligo-dT RNA-seq does due to the carry-over of rRNAs [1,6,7]. Therefore, an ideal method for rRNA removal should be simplistic, efficient, reliable and yet cost-effective so it can be easily adapted for homemade RNA-seq library construction.

Here, we developed a novel reverse transcriptase-mediated ribosomal RNA depletion (RTR2D) strategy. We demonstrated that the RTR2D method was simple and efficient, and depleted human and mouse rRNAs with high specificity without affecting mRNA and noncoding RNA transcripts. RNA-seq data analysis indicated that the RTR2D method yielded highly correlative transcriptomic landscape with that of the commonly-used NEBNext rRNA Depletion Kit at both mRNA and lncRNA levels. In a proof-of-principle study of determining the transcriptomic response to MDM2 inhibitor in human osteosarcoma cells, we found that the RNA-seq dataset from the RTR2D-depleted rRNA samples identified more differentially expressed mRNA and lncRNA transcripts than that from the NEBNext rRNA Depletion samples, suggesting that RTR2D may have lower off-target depletion of non-rRNA transcripts. Thus, the reported RTR2D should be a valuable tool to deplete rRNAs for RNA-seq library constructions.

**Materials and methods***Cell culture, chemicals, and enzymes*

Human breast cancer line MCF-7 and human osteosarcoma line SJSA1 were kindly provided by Dr. Olufunmilayo Olopade of The University of Chicago and Dr. Carl G. Maki of Rush University Medical Center, respectively. Mouse line iMEF cells are immortalized mouse embryonic fibroblasts as previously characterized [8,9]. All above lines were maintained in complete DMEM supplemented with 10% fetal bovine serum (FBS, Gemini Bio-Products, West Sacramento, CA), containing 100 U/ml penicillin and 100 µg/ml streptomycin at 37 °C in 5% CO<sub>2</sub> as described [10–12]. M–MuLV reverse transcriptase, ProtoScript<sup>®</sup> II Reverse Transcriptase, WarmStart RTx Reverse Transcriptase, RNase H, DNase I, Exonuclease I, Murine RNase Inhibitor, NEBNext<sup>®</sup> rRNA Depletion Kit (Human/Mouse/Rat), and NEBNext Ultra Directional RNA Library

Prep Kit for Illumina were purchased from New England Biolabs (NEB, Ipswich, MA). The Ribo-Zero Gold rRNA Removal Kit (Human/Mouse/Rat) was purchased from Illumina (San Diego, CA). Nutlin3A was purchased from Selleckchem (Houston, TX). Unless indicated otherwise, all other chemicals were purchased from Sigma Millipore (St. Louis, MO) or Thermo Fisher Scientific (Waltham, MA, US).

#### *Design and synthesis of oligonucleotide probes for rRNA-specific reverse transcription (RT)*

The design and locations of RT probes for rRNAs are shown in **Suppl. Fig. S2B**. All DNA oligonucleotides were synthesized by Sigma Millipore as previously described [13,14]. The full-length sequences of the oligo probes are listed in **Suppl. Table S1**.

#### *Total RNA isolation, RNA integrity and quantitative analysis*

Exponentially growing MCF7 or iMEF cells, or SASJ1 cells treated with or without Nutlin3A were subjected to total RNA isolation by using NucleoZOL RNA Isolation kit (Takara Bio USA, Mountain View, CA) according to the manufacturer's instructions as described [15,16]. RNA integrity and quantity were assessed with an Agilent 2100 Bioanalyzer (Santa Clara, CA). Briefly, RNA samples (1.0  $\mu$ l) were loaded onto the Bioanalyzer RNA Nano Chips, along with size marker and subjected to electrophoresis according to the manufacturer's instructions. Both gel images and electropherograms were obtained to assess the integrity and quantity of RNA samples. For quick and effective assessment of the quality of total RNA, we also ran 1% agarose gel with 1% bleach as previously described [17].

#### *Reverse transcription (RT)-based ribosomal RNA depletion (RTR2D)*

The detailed protocol for performing the RTR2D procedure is described in the **Suppl. Methods**. Briefly, human and mouse total RNA was first subjected to RNA integrity analysis using Agilent 2100 Bioanalyzer (usually RNA Integrity Number or RIN  $\geq$  8). One microgram of total RNA (at 100–500 ng/ $\mu$ l) was used for rRNA removal and subsequent RNA-seq library preparation. For the removal of all rRNAs (human and mouse), the 30 oligo probes were pooled at a pre-optimized ratio to make the rRNA probe mix at a final overall concentration of 1  $\mu$ g/ $\mu$ l. To specifically hybridize the probes to rRNAs, total RNA (1  $\mu$ g) was mixed with 6  $\mu$ l probes in 20  $\mu$ l total volume and subjected to a touchdown annealing protocol: 85°C  $\times$  10', x 47 cycles with  $-1^\circ\text{C}/\text{cycle}$ . For the removal of individual rRNAs, 1  $\mu$ g of rRNA-specific probe was used for annealing. The reverse transcription was carried out in 50  $\mu$ l reaction at 37 °C for 60 min as follows: to the 20  $\mu$ l annealed probes/rRNA mix, added 1  $\mu$ l of RNase Inhibitor, 5  $\mu$ l of 10x RT buffer (NEB), 5  $\mu$ l of 10 mM dNTPs, 1  $\mu$ l of M–MuLV reverse transcriptase, and 18  $\mu$ l RNase-free ddH<sub>2</sub>O. At the end of reverse transcription, excess probes were removed by adding Exonuclease I to the reaction mix and incubating at 37 °C for 30 min. The RT reaction products were cleaned up with PC-8 extractions, followed by ethanol precipitation. The pellet was then resuspended in 42  $\mu$ l of RNase-free ddH<sub>2</sub>O, added with 1  $\mu$ l of RNase Inhibitor, 5  $\mu$ l of RNase H 10x Reaction Buffer and 2  $\mu$ l of RNase H, and incubated at 37 °C for 30 min. At the end of the incubation, the reaction mix was added with 5  $\mu$ l of 10x DNase I Reaction Buffer, 43  $\mu$ l of RNase-free ddH<sub>2</sub>O and 2  $\mu$ l of DNase I and incubated at 37 °C for 30 min. The reaction was terminated by PC-8 extractions and ethanol precipitation. The rRNA-depleted pellet was resuspended in RNase-free ddH<sub>2</sub>O and used for subsequent RNA-seq library preparation.

For the methodology controls, rRNA depleted samples were also prepared by using the NEBNext® rRNA Depletion Kit and/or the

Ribo-Zero Gold rRNA Removal Kit by following the manufacturer's instructions.

#### *RNA-seq library preparation, next-generation sequencing (NGS) and NGS data analysis*

All RNA sequencing libraries were constructed by using the NEBNext Ultra Directional RNA Library Prep Kit for Illumina according to the manufacturer's instructions, and sequenced in parallel on the Illumina HiSeq 4000 instrument. RNA-seq data were processed by using the TopHat2 and Cufflinks programs for read mapping and transcript assembly and quantification. FPKM (Fragments Per Kilobase of transcript per Million mapped reads) correlation analysis was carried out to determine transcript expression correlation between RTR2D and NEB's kit depleted libraries. Differential gene expression was analyzed using the DESeq2 package [18]. The NGS dataset was deposited in the NCBI Sequence

Read Archive (SRA) under accession number # PRJNA574772 (<https://www.ncbi.nlm.nih.gov/sra/PRJNA574772>).

#### *Touchdown-quantitative real-time PCR (TqPCR)*

The TqPCR was carried out as described [13,14,19]. Briefly, total RNA or rRNA-depleted samples were subjected to RT reactions using hexamer and M–MuLV Reverse Transcriptase. The RT/cDNA products were further diluted and used as PCR templates. The qPCR primers were designed with Primer3 Plus program (**Suppl. Table S2**), and the qPCR analysis was carried out using the 2x SYBR Green qPCR kit (Bimake, Houston, TX) with our previously optimized TqPCR protocol [19]. All reactions were done in triplicate. *GADPH* was used as a reference gene. All sample values were normalized to *GADPH* expression by using the  $2^{-\Delta\Delta\text{Ct}}$  method as described [12,20,21].

#### *Statistical analysis*

The sample size was not predetermined by any statistical methods, and investigators were not blinded to sample allocation for most of the experiments. All quantitative studies were carried out in triplicate and/or performed in three independent batches. Microsoft Excel program (Redmond, WA, USA) was used to calculate standard deviation (S.D.). Data in all graphs are presented as the mean of either independent biological or technical replicates, as indicated in the figure legends, with the error bars representing standard deviation. Pearson's correlation coefficient ( $R^2$ ) was calculated by linear regression analysis. Statistically significant differences between samples were determined by one-way analysis of variance. A value of  $p < 0.05$  was considered statistically significant when one comparison was being made.

## **Results and discussion**

### *Reverse transcription-based removal may represent a novel and effective approach to rRNA depletion*

With the even-increasing demands on analyzing transcriptomic landscapes using RNA-seq next-generation sequencing technology, it is important to have a simple, reliable, cost-effective, and user-friendly technique to prepare RNA-seq libraries, in which the removal of rRNA is one of the most critical prerequisites. While there are numerous methods used to remove rRNAs for RNA-seq library preparation [22], at least three types of methodologies have been commonly used to separate rRNAs from other transcripts (mainly mRNAs and noncoding RNAs) (**Suppl. Fig. 1**)\*\*. First, the oligo d(T)-based selection and exome probe capture are



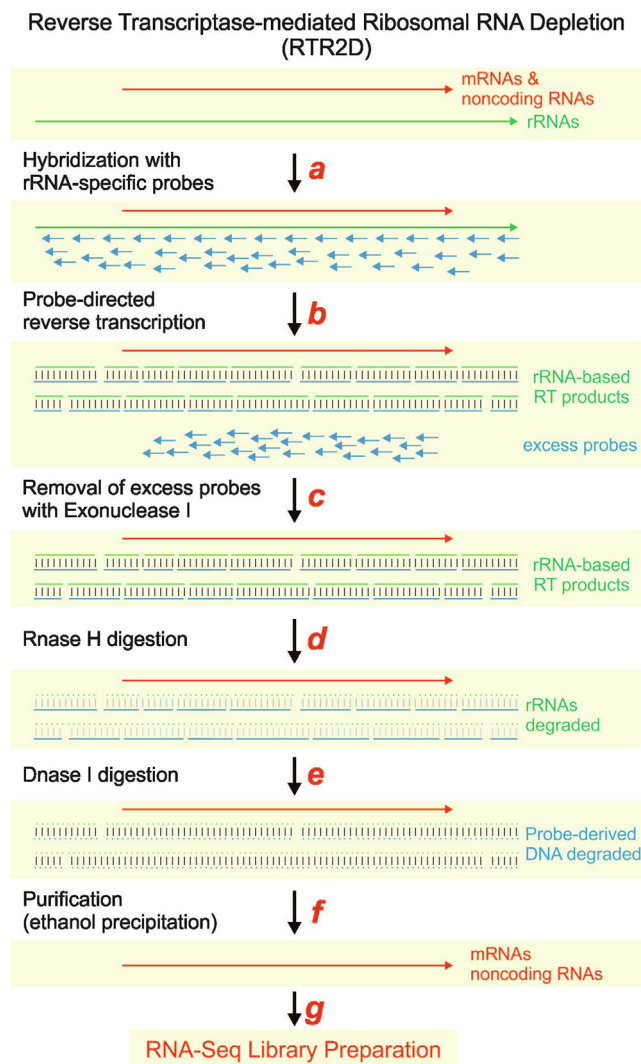
commonly-used methods to purify mRNAs or exosomal transcripts away from rRNAs by using biotinylated probes to bind to streptavidin-beads (Suppl. Fig. S1A). An obvious shortcoming of oligo d(T) selection is the loss of non-poly A tailed transcripts, including most of the noncoding RNAs. A second commonly-used method is to pull-down rRNAs directly with the use of biotinylated rRNA-targeting probes to bind to streptavidin-beads (Suppl. Fig. S1B). Representative commercial kits include the Rib-Zero from Illumina and RiboMinus from Qiagen. While this approach should theoretically leave mRNAs and noncoding RNAs intact, the pulldown efficiencies vary significantly among samples. This approach also prefers the samples with high RNA integrity, which may not be attainable when RNA samples are prepared from clinical samples. A third approach is to take advantage of the feature of RNase H-mediated degradation of RNA:DNA hybrids by using a large panel of overlapping DNA oligo probes that are complementary to rRNAs (Suppl. Fig. S1B). A representative commercial kit is

the NEBNext® rRNA Depletion Kit. In fact, this approach has been shown highly effective in depleting rRNAs from human formalin-fixed paraffin-embedded samples [3,22]. In our pilot studies, we compared the rRNA depletion efficiency between the Illumina's Ribo-Zero Gold rRNA Removal Kit and the NEBNext® rRNA Depletion Kit, and found that NEBNext® rRNA Depletion Kit was superior to the Ribo-Zero Gold rRNA Removal Kit in terms of rRNA removal efficiency, reproducibility and low off-target depletion of non-rRNA transcripts. An ideal rRNA depletion system should be simple, effective, inexpensive and user-friendly.

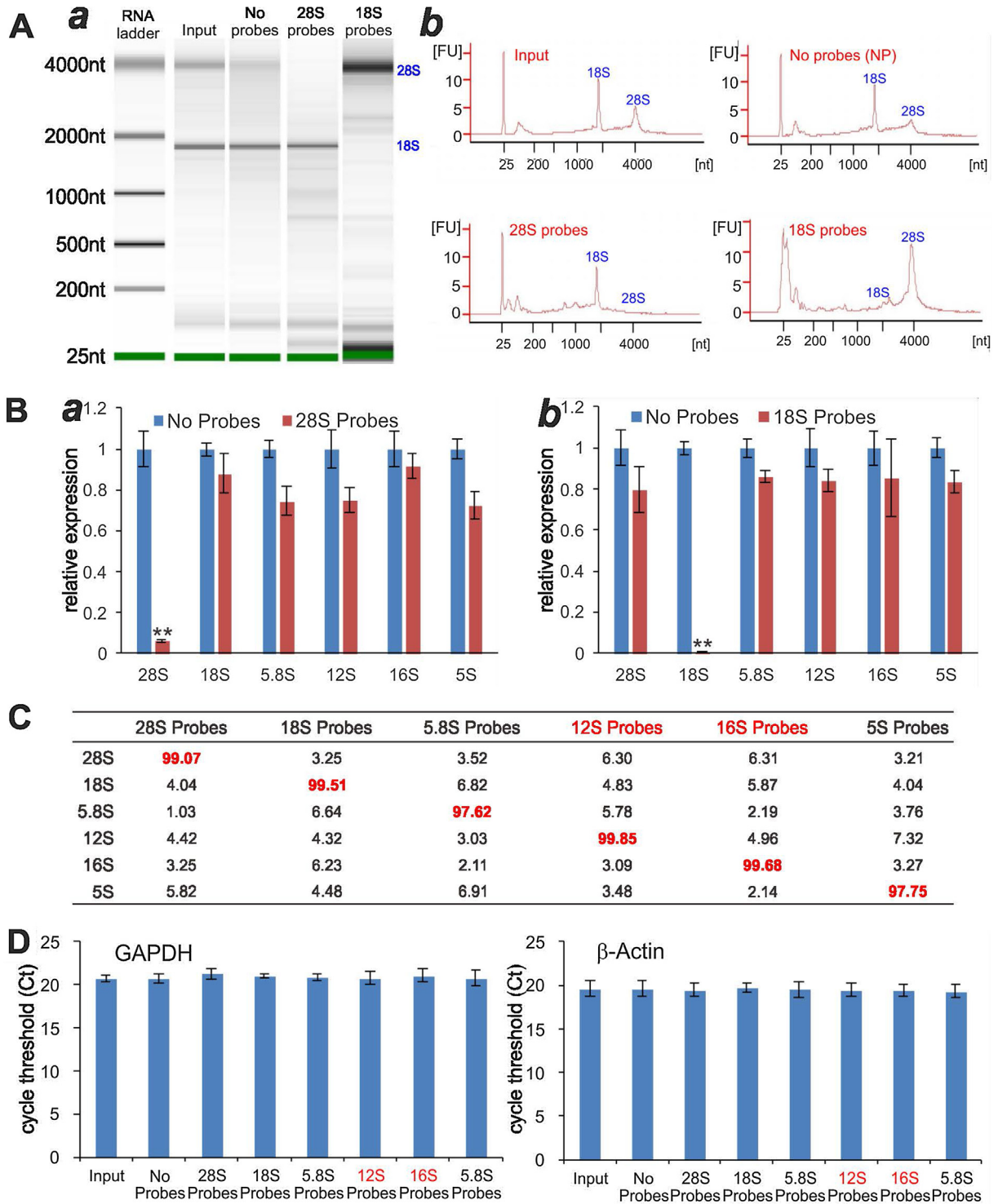
To that end, we devised a reverse transcription-directed ribosomal RNA depletion (RTR2D) system as a means to remove rRNAs and mitochondrial RNAs. Specifically, in this system, based on the homology alignments of human and mouse sequences (Suppl. Fig. S2A), a total of 30 oligonucleotide probes complementary to human and mouse rRNAs (i.e., 28S, 18S, 5.8S, and 5S RNAs) and mitochondrial RNAs (i.e., 16S and 12S RNAs), spacing approximately 400 ~ 500nt, were synthesized (Suppl. Fig. S2B, a & b; Suppl. Table S1). Thanks to the high sequence homology, 24 of the 30 probes can be shared for both human and mouse samples, and only three human or mouse-specific probes (one each for 28S, 12S and 16S RNA) were synthesized (Suppl. Fig. S2B, b). These probes were pooled at an optimized ratio (Suppl. Methods), and then hybridized to the rRNAs in a touchdown fashion, followed by reverse transcription reaction (Fig. 1, a & b). After the excessive probes were removed by Exonuclease I digestion (Fig. 1, c), the reaction mix was subjected to RNase H digestion (Fig. 1, d), followed by DNase I digestion to degrade the DNA portion of the RT products (Fig. 1, e). The rRNA-depleted sample was then subjected to PC-8 extraction and ethanol precipitation, and used for RNA-seq library preparation (Fig. 1, f & g).

*The RTR2D system specifically depletes individual rRNAs with high efficiency*

We next carried out the proof-of-principle experiments to test whether specific rRNAs can be effectively removed by rRNA-specific probes from total RNA without significant off-target depletion. We used human total RNA and performed RT-based removal of individual rRNAs with respective RT probes, and found that 28S and 18S rRNAs were effectively and specifically depleted with 28S-specific and 18S-specific probes, respectively, as assessed by the Agilent 2100 Bioanalyzer (Fig. 2A, a & b). Quantitative qPCR analysis revealed that 28S-specific RT probes specifically decreased the expression of 28S without affecting other rRNAs (Fig. 2B, a). Similarly, the 18S-specific probes were shown to effectively and specifically remove 18S rRNA in the total RNA sample (Fig. 2B, b). Similar experiments were carried out to assess the removal efficiency and specificity of 5.8S, 5S, 12S and 16S-specific probes, and all of them were shown to effectively deplete the respective rRNAs (Suppl. Fig. S3A, ab), and their depletions were probe-specific and did not affect other rRNAs or mitochondrial RNAs (Suppl. Fig. S3B, a–d). Based on the results from TqPCR analysis, the RT probes specific for 28S, 18S, 5.8S, 12S, 16S and 5S RNAs accomplished the removal rates of 99.07%, 99.51%, 97.62%, 99.85%, 99.68, and 97.75% for corresponding rRNAs, respectively (Fig. 2C). Furthermore, we assessed the potential off-target removal of non-rRNA transcripts, i.e., mRNA, and found that, compared with the input or no probes control, all rRNA-specific RT probes did not cause any significant losses in the expression of mRNA, such as house-keeping genes *GAPDH* and *β-ACTIN* (Fig. 2D). Similar results were obtained from the experiments using mouse total RNA samples (data not shown). Collectively, these results demonstrate the feasibility of using RT-based approach to the rRNA removal in human and mouse total RNA samples.



**Fig. 1. The schematic representation of the workflow for the reverse transcriptase-mediated ribosomal RNA depletion (RTR2D) strategy.** Total RNA (usually 0.5–1.0 μg) is incubated and hybridized with a panel of 30 (human & mouse) rRNA-specific DNA oligo probes (a), followed by reverse transcription (RT) (b). After the removal of excess oligo probes with Exonuclease I (c), the resultant RT products are subjected to RNase H digestion to degrade the rRNA portions of the RNA:DNA hybrid (d), and then the DNA components are degraded by DNase I (e). The intact mRNAs and noncoding RNAs are subsequently purified by ethanol precipitation (f) and subjected to RNA-seq library construction (g). The locations and sequences of individual rRNA-specific probes are shown in Suppl. Fig. S2 and Suppl. Table S1.



**Fig. 2. Specificity and efficiency of the designed rRNA-specific probes.** (A) Removal efficiency and specificity of 28S and 18S rRNA-specific probes. Human total RNA (1.0 μg) was subjected to the RTR2D procedure and analyzed with an Agilent 2100 Bioanalyzer. The representative gel image (a) or electropherograms (b) are shown. “Input” and “No probes (NP)” groups were used as controls. (B) Quantitative analysis of rRNA expression profiles after 28S rRNA (a) or 18S rRNA (b) specific probe mediated removal. Mitochondrial rRNAs 12S and 16S were also included in the studies. “\*\*\*\*” p < 0.01 compared with that of the NP group’s. (C) Removal efficiencies for the probes designed for individual rRNAs. (D) Effect of the RTR2D procedure using individual rRNA probe sets on the expression of housekeeping genes GAPDH and β-ACTIN as assessed by TqPCR. All qPCR reactions were done in triplicate.

The RTR2D-based rRNA removal is as effective as that of the NEBNext rRNA depletion system

We next sought to test the rRNA removal efficiency and specificity of the pooled RT probes by comparing with a commonly used

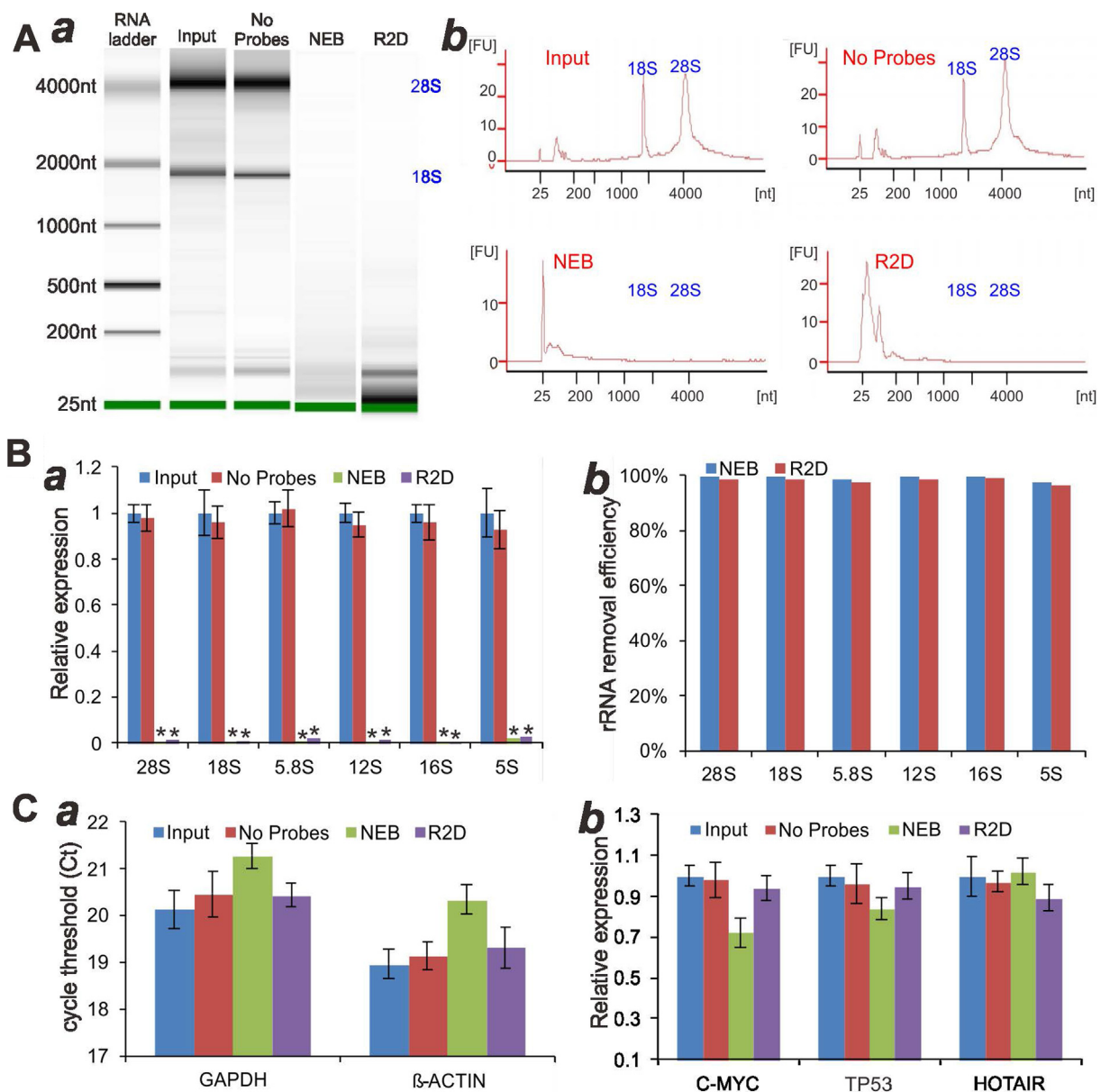
commercial rRNA removal kit. As mentioned above, in our pilot comparison studies we found that, regardless of the RNA integrity, the NEBNext rRNA Depletion Kit (referred to as NEB kit, thereafter) consistently out-performed the rRNA-based oligo pulldown kits, such as the Rib-Zero from Illumina and RiboMinus from Qiagen.

Therefore, we compared the rRNA removal features of the RTR2D system with those of the NEB kit's.

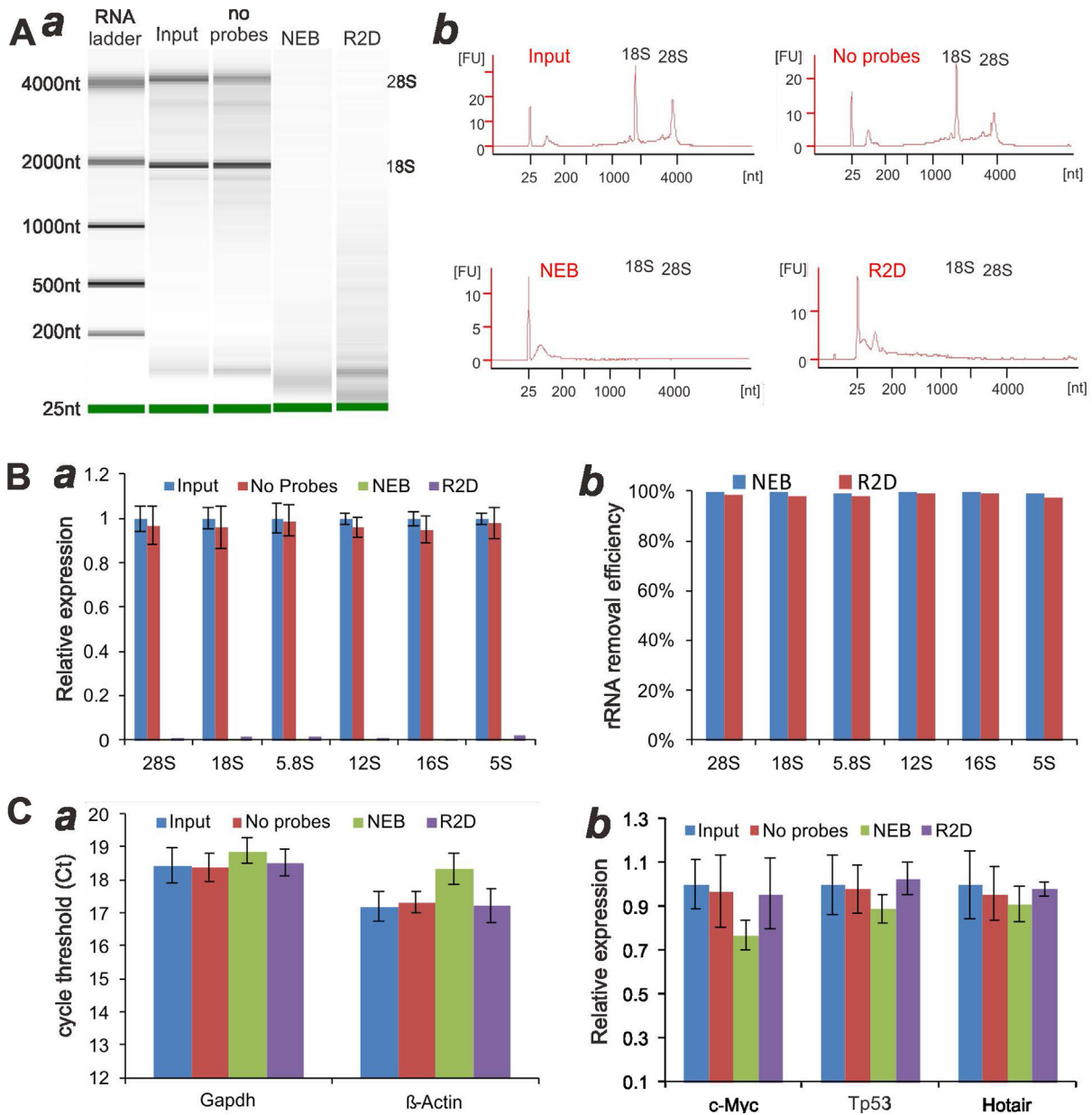
We first conducted extensive preliminary experiments and determined the relative molar ratios of the 30 RT probes listed in **Suppl. Table S1**. As shown in the **Suppl. Methods**, the 30 RT oligo probes were pooled at the optimized ratios. Using 1  $\mu\text{g}$  of human total RNA we showed that the RTR2D system depleted 28S and 18S rRNAs as effectively as that of the NEB kit as shown on Bioanalyzer gel image and electropherograms, compared with the "Input" or "no probes" control (**Fig. 3A, a & b**). Quantitative TqPCR analysis indicates that the four rRNAs and two mitochondrial RNAs were effectively removed from the RNA sample with the removal rates > 99.0% for all rRNAs (except for 5S at 97.8%) (**Fig. 3B, a & b**). Furthermore, quantitative TqPCR analysis revealed that the RTR2D system exhibited very low off-target depletion effects on

the abundant housekeeping genes, such as *GAPDH* and  $\beta$ -*ACTIN*, mRNAs with average abundance such as *c-MYC* and *TP53*, and long noncoding RNA (lncRNA) *HOTAIR* (**Fig. 3C, a & b**).

Similarly, we applied the RTR2D system to mouse samples and found that the RTR2D system depleted 28S and 18S rRNAs as effectively as that of the NEB kit (**Fig. 4A, a & b**). Quantitative TqPCR analysis revealed that the rRNAs were effectively removed from the RNA sample with the removal rates > 99.0% for all rRNAs (**Fig. 4B, a & b**), while the RTR2D system did not exhibit any significant off-target depletion effects on the abundant housekeeping genes, such as *Gapdh* and  $\beta$ -*Actin*, mRNAs with average abundance such as *c-Myc* and *Tp53*, and long noncoding RNA (lncRNA) *Hotair* (**Fig. 4C, a & b**). Collectively, these results demonstrate that the RTR2D system can deplete both human and mouse rRNAs with high efficiency and specificity, which is comparable



**Fig. 3. Comparison of human rRNA removal specificity and efficiency between the RTR2D procedure and NEBNext® rRNA Depletion kit.** Human total RNA (1.0  $\mu\text{g}$ ) was subjected to the RTR2D (R2D) (with the pooled rRNA probes) or the NEBNext® rRNA Depletion (NEB) protocol. The rRNA-depleted products were subjected to the Bioanalyzer. Representative gel image (**a**) and electropherograms (**b**) are shown. "Input" and "No probes (NP)" groups were used as controls. (**B**) TqPCR analysis of the residual rRNA species (**a**) and removal efficiency (**b**). "\*\*\*\*"  $p < 0.001$ , compared with that of the NP group or the Input group. (**C**) Effect of rRNA removal protocols on the expression of housekeeping genes *GAPDH* and  $\beta$ -*ACTIN* (**a**) and genes/lncRNA with different abundances, *c-MYC*, *TP53* and lncRNA *HOTAIR* (**b**). All qPCR reactions were done in triplicate.



**Fig. 4.** Comparison of mouse rRNA removal specificity and efficiency between the RTR2D procedure and NEB Next rRNA Depletion kit. Mouse total RNA (1.0  $\mu$ g) was subjected to the RTR2D (R2D) or the NEBNext<sup>®</sup> rRNA Depletion (NEB) protocol. The rRNA-depleted products were subjected to the Bioanalyzer. Representative gel image (**a**) and electropherograms (**b**) are shown. “Input” and “No probes (NP)” groups were used as controls. (**B**) TqPCR analysis of the residual rRNA species (**a**) and removal efficiency (**b**). “\*\*\*\*”  $p < 0.001$ , compared with that of the NP group or the Input group. (**C**) Effect of rRNA removal protocols on the expression of housekeeping genes *Gapdh* and  $\beta$ -*Actin* (**a**) and genes/lncRNA with different abundances, *c-Myc*, *Tp53* and lncRNA *Hotair* (**b**). All qPCR reactions were done in triplicate.

with the results obtained from the commonly-used NEB rRNA Depletion Kit.

*The rRNA depletion efficiency of the RTR2D system can be affected by RT reaction temperature, the quantity of RT probes, and the removal of excess probes*

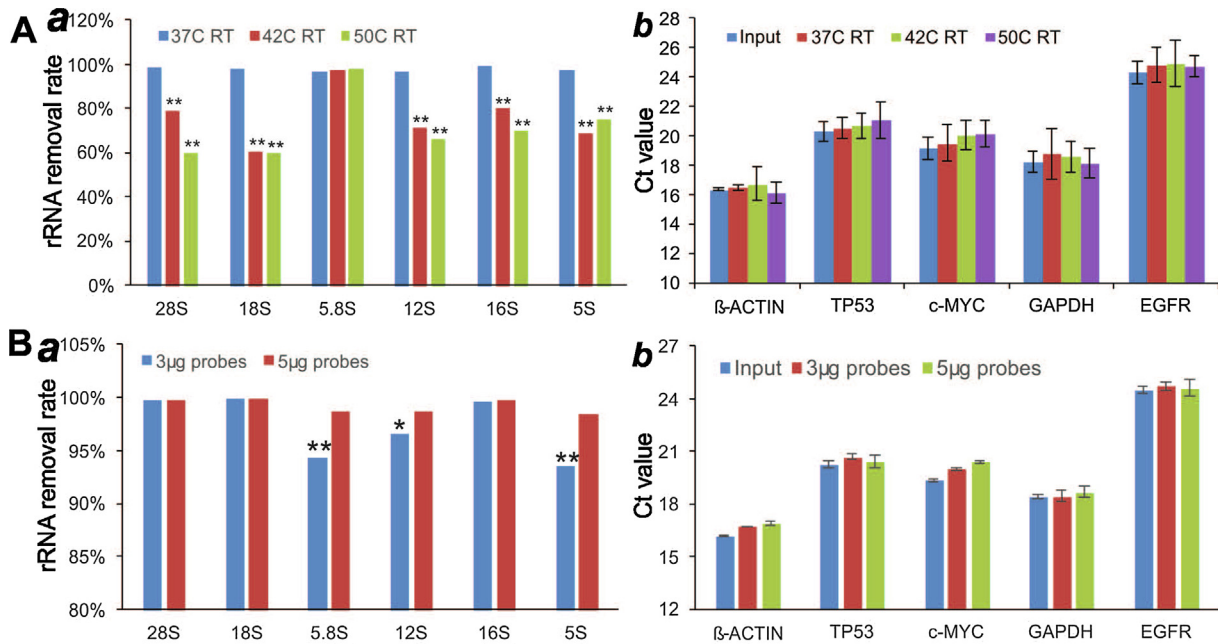
While the above experiments proved the feasibility of using the RTR2D system as a simple and efficient means to deplete rRNAs, some of the essential parameters must be optimized prior to its routine use for RNA-seq preparations. Thus, we further optimized the reverse transcription reaction conditions in order to achieve more efficient and reproducible rRNA depletion.

It is conceivable that reverse transcription reactions carried out at higher temperatures may lead to better rRNA depletion effi-

ciency and specificity. To test this possibility, we compared the rRNA removal efficiency by using the ProtoScript<sup>®</sup> II Reverse Transcriptase (42 °C) and WarmStart RTx Reverse Transcriptase (50 °C), along with M-MuLV reverse transcriptase (37 °C). Surprisingly, we found that, under the same conditions except RT reaction temperature, M-MuLV reverse transcriptase mediated the most efficient rRNA depletion at 37 °C, while RT reactions carried out at 42 °C and 50 °C exhibited significantly lower depletion rates for most of the rRNAs (except 5.8S rRNA) (Fig. 5A, a). Nonetheless, the off-target depletion of mRNA transcripts, such as  $\beta$ -*ACTIN*, *TP53*, *c-MYC*, *GAPDH* and *EGFR*, was not presented under all three tested reaction temperatures (Fig. 5A, b). Thus, the RT reactions should be performed by using M-MuLV reverse transcriptase at 37 °C.

The use of sufficient rRNA-specific probes for reverse transcription may be an important parameter to ensure effective depletion





**Fig. 5. Optimization of the reverse transcription conditions for the RTR2D protocol.** (A) Effect of different reverse transcriptase and reaction temperatures on rRNA removal efficiency and specificity. Human total RNA (1.0 μg) was subjected to the RTR2D procedure under the same condition, except the use of different RT enzymes and reaction temperatures as follows: 37 °C (M–MuLV reverse transcriptase), 42 °C (ProtoScript® II Reverse Transcriptase), and 50 °C (WarmStart RTx Reverse Transcriptase). The Input and NP groups were used as controls. The expression levels of rRNAs were determined by TqPCR. \*\*\*\* $p < 0.01$  when compared with that of the NP or Input group (a). The expression of several representative genes was also determined by TqPCR (b). (B) Effect of probe quantities on rRNA removal efficiency and specificity. Human total RNA (1.0 μg) was subjected to the RTR2D procedure under the same condition, except the use of 3 μg or 5 μg of the pooled rRNA probes for the RT reaction. The expression levels of rRNAs were determined by TqPCR (a). \*\*\*\* $p < 0.05$ , \*\*\*\*\* $p < 0.01$  when compared with that of the NP and Input groups. The expression of several representative genes was also determined by TqPCR (b). All qPCR reactions were done in triplicate.

of the target RNAs. We compared the rRNA removal efficiency and specificity by performing the RTR2D procedure with 3 μg and 5 μg of the pooled probes. We found that, while the 3 μg and 5 μg probe groups yielded similar depletion efficiencies for 28S, 18S and 16S RNAs ( $p > 0.1$ ), the 3 μg probe group exhibited detectable decrease in depletion rates for 5.8S, 12S and 5S RNAs, compared with that of the 5 μg probe group ( $p < 0.05$ ) (Fig. 5B, a). Furthermore, the 5 μg probe group did not cause any off-target depletion of representative mRNA transcripts (Fig. 5B, b). These results suggest that sufficient probes should be used for the RT-based rRNA depletion. We used 6 μg probes per RT reaction for the following RNA-seq library preparations.

We also analyzed the effect of excess probes on rRNA depletion efficiency. We showed that, while the gross gel image and electropherograms did not show significant differences in the group without excess probe removal, compared with that of the probe removal group (Fig. 6A, a), the presence of excess probes after RT reaction slightly but consistently decreased the rRNA depletion efficiency, especially for 18S, 5.8S and 12S RNA (Fig. 6B, a), although the excess probes did not lead to any significant off-target depletion of the representative mRNA transcripts (Fig. 6B, b). Thus, we routinely included the Exonuclease I-mediated excess probe removal step in the RTR2D protocol.

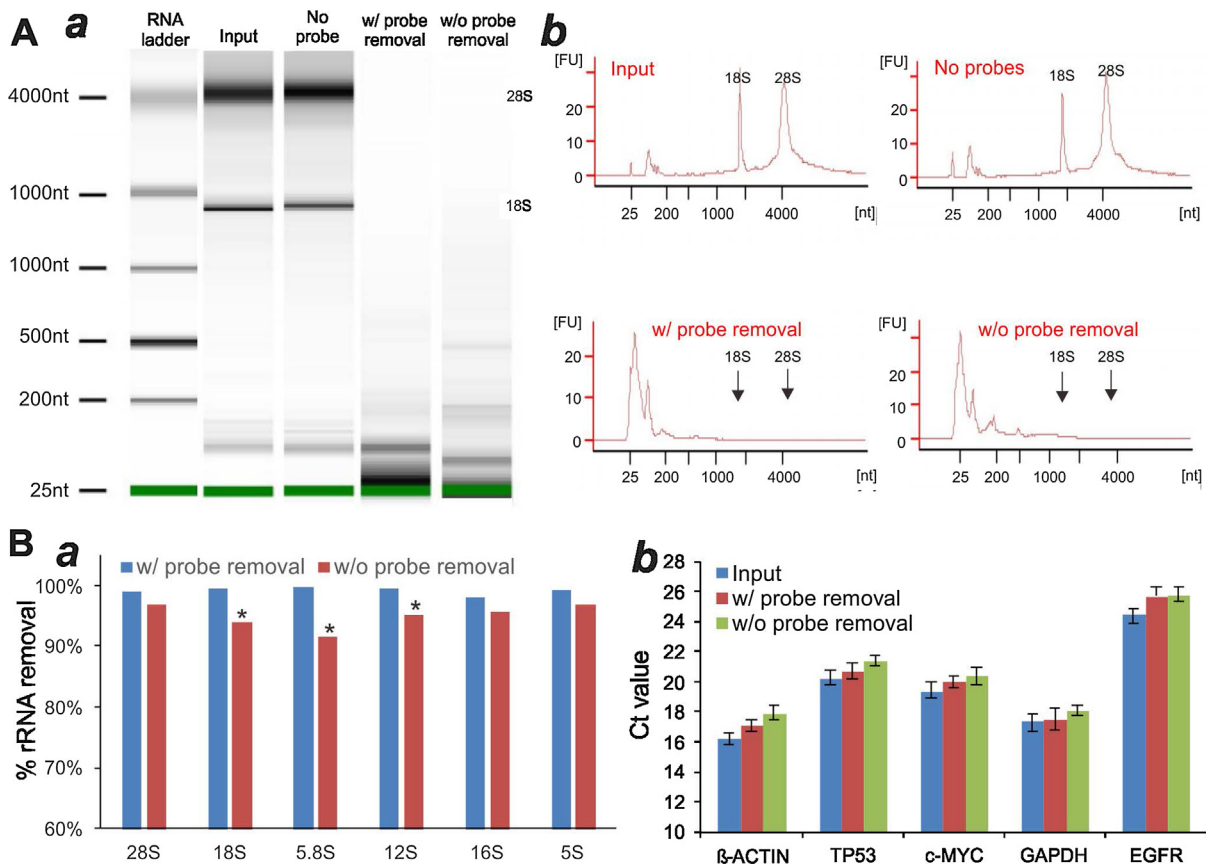
*RNA-seq analysis reveals that the transcriptomic profiles of the RTR2D-depleted samples are comparable with those of the NEB kit-depleted samples*

We further analyzed the RNA-seq data quality of the libraries prepared from the RTR2D-based rRNA-depleted samples, compared with those prepared from the NEBNext rRNA Depletion Kit. Using the RNA samples isolated from a human osteosarcoma line

and rRNA-depleted with RTR2D protocol or the NEBNext protocol, we found that the overall percentages of different transcript categories were similar for both rRNA-depleted samples prepared with the RTR2D and NEBNext protocols (Fig. 7A, a vs. b). Scatter plot comparison analysis indicated that the expression levels of mRNA transcripts prepared with the RTR2D protocol and the NEBNext protocol were highly correlative in replicate experiments ( $R = 0.94$ ) (Fig. 7B, a & c). Similarly, the expression levels of lncRNA transcripts prepared with the RTR2D protocol and the NEBNext protocol were also correlative in replicate experiments ( $R = 0.86$  and  $0.85$ ) (Fig. 7C, a & c), although the correlation coefficients are slightly lower than that of the mRNA transcripts. The differences of correlation coefficients for mRNAs vs. lncRNAs may be explained by their differences in expression levels, and lower expression levels of lncRNAs may lead to higher variations in detection and thus lower correlations. Nonetheless, the NGS replicate studies strongly indicate that RNA-seq libraries prepared from the RNA samples using the RTR2D rRNA depletion protocol exhibited similar transcriptomic landscape.

We further compared the transcriptomic landscape in response to MDM2 inhibitor Nutlin3A in human osteosarcoma line SJS1 cells based on the RNA-seq analysis of the libraries prepared with the RTR2D protocol vs. the NEBNext protocol. Upon Nutlin3A treatment, we found that, while the NEBNext protocol yielded 295 up-regulated and 343 down-regulated mRNA transcripts (Fig. 8A, a), the RTR2D protocol produced 539 up-regulated and 646 down-regulated mRNA transcripts (Fig. 8A, b), suggesting that the RTR2D-based rRNA depletion process may preserve mRNA and lncRNA transcripts more effectively and thus lead to the identification of more differentially expressed transcripts. Using more stringent criteria, we found that 25 transcripts were up-regulated in the RNA-seq libraries constructed by both protocol (Fig. 8A, c) and 32





**Fig. 6.** Effect of excess probes on rRNA removal efficiency. (A) The rRNA removal efficiency assessed by the Bioanalyzer. Human total RNA (1.0  $\mu$ g) was subjected to the RTR2D procedure under the same condition, except that the excess rRNA probes were either degraded with Exonuclease I or left intact without the use of Exonuclease I. The rRNA-depleted products were subjected to the Bioanalyzer. Representative gel image (a) and electropherograms (b) are shown. "Input" and "No probes (NP)" groups were used as controls. (B) The expression levels of rRNAs (a) and several representative genes (b) were determined by TqPCR. "\*"  $p < 0.05$  when compared with that of the NP and Input groups. All qPCR reactions were done in triplicate.

transcripts were down-regulated in the RNA-seq libraries constructed by both protocol (Fig. 8A, d), with significantly more differentially expressed mRNA transcripts in the RNA-seq libraries with the RTR2D protocol.

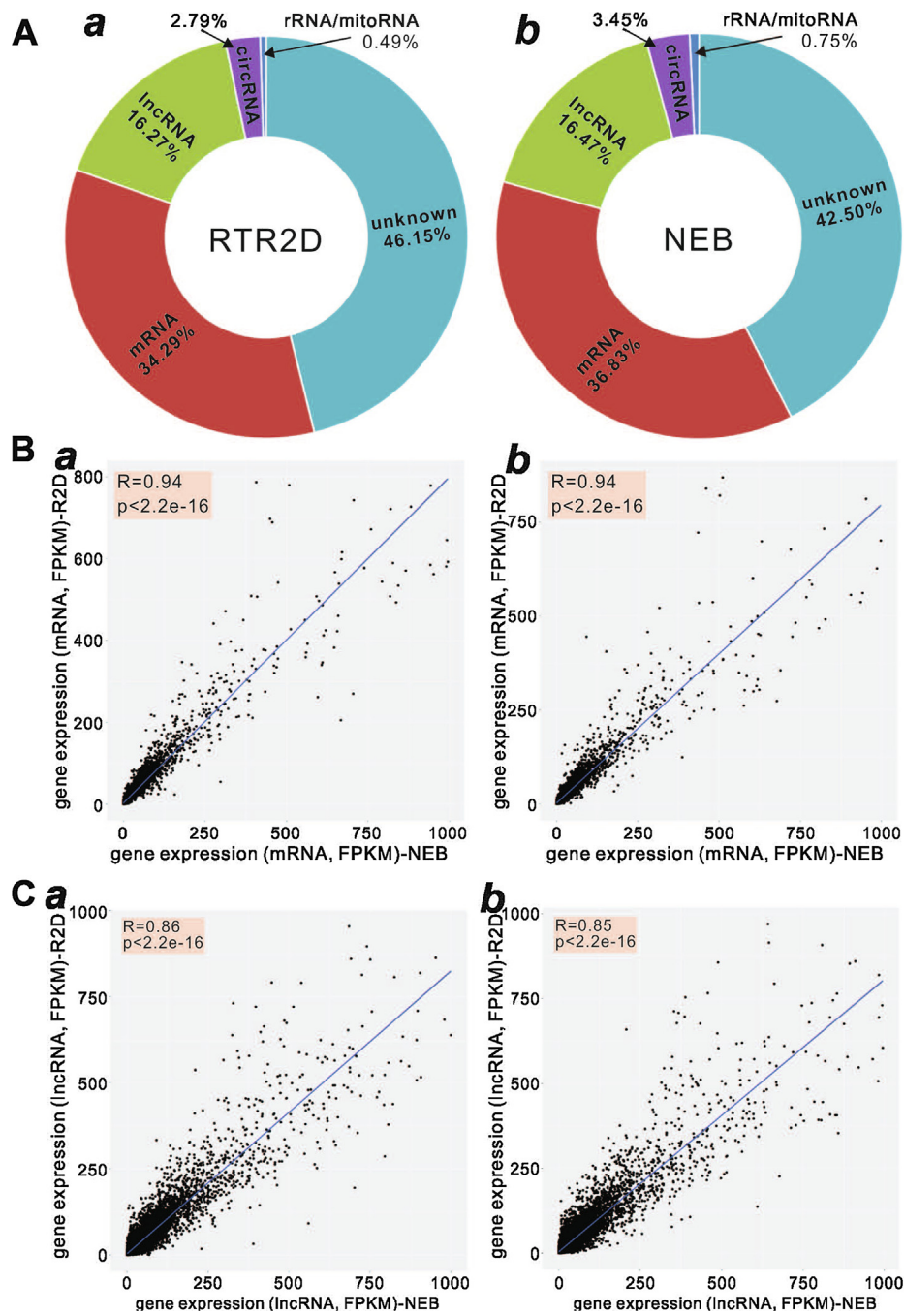
Furthermore, we found that 163 up-regulated and 157 down-regulated lncRNAs were identified in the RNA-seq libraries prepared with the NEBNext protocol (Fig. 8B, a), whereas 258 up-regulated and 249 down-regulated lncRNAs were found in the RNA-seq libraries constructed with the RTR2D protocol (Fig. 8B, b), with more differentially expressed lncRNAs identified in the RNA-seq libraries with the RTR2D protocol. Using more stringent criteria, we found that 38 lncRNAs were up-regulated in the RNA-seq libraries constructed by both protocol (Fig. 8A, c) and 11 lncRNAs were down-regulated in the RNA-seq libraries constructed by both protocol (Fig. 8A, d). Collectively, the genomewide transcriptomic analysis demonstrates that the RTR2D protocol can effectively deplete rRNAs and yield high quality transcriptomic analysis data, which are comparable with, if not better than, that obtained from commercial kits, such as the NEBNext rRNA Depletion kit.

*A simplified, reliable and cost-effective rRNA removal technique should significantly facilitate whole transcriptome analyses in biomedical research*

Since the advent of next-generation sequencing technology in early 2000's, RNA-seq-based whole transcriptome analysis has

become a routine for many transcriptome landscape studies. As the rRNA removal is a prerequisite for the vast majority of RNA-seq analyses, many techniques have been developed to accomplish this purpose, most of which perform well when high-quantity and high-quality total RNA samples are used [22,23], although some of the early methods are quite complex and less frequently used. However, methods with high efficiency has to be used to overcome the challenges of low-quality and/or low-quantity RNA samples. Representatives of these methods include the RNase H-based selective depletion of abundant RNA (SDRNA) [3], Ribo-Zero [24], the NuGEN Ovation RNA-seq system [25], and template-switching mechanism at the 5' end of the RNA template (SMART) [26]. Several comparison studies indicate each method has distinct merits, and their suitability should requires a careful comparison of multiple metrics for a given project [2,27,28]. In our pilot studies, the SDRNA-based NEBNext rRNA Depletion Kit outperformed the popular Ribo-Zero kits regardless of RNA quantity and quality [22].

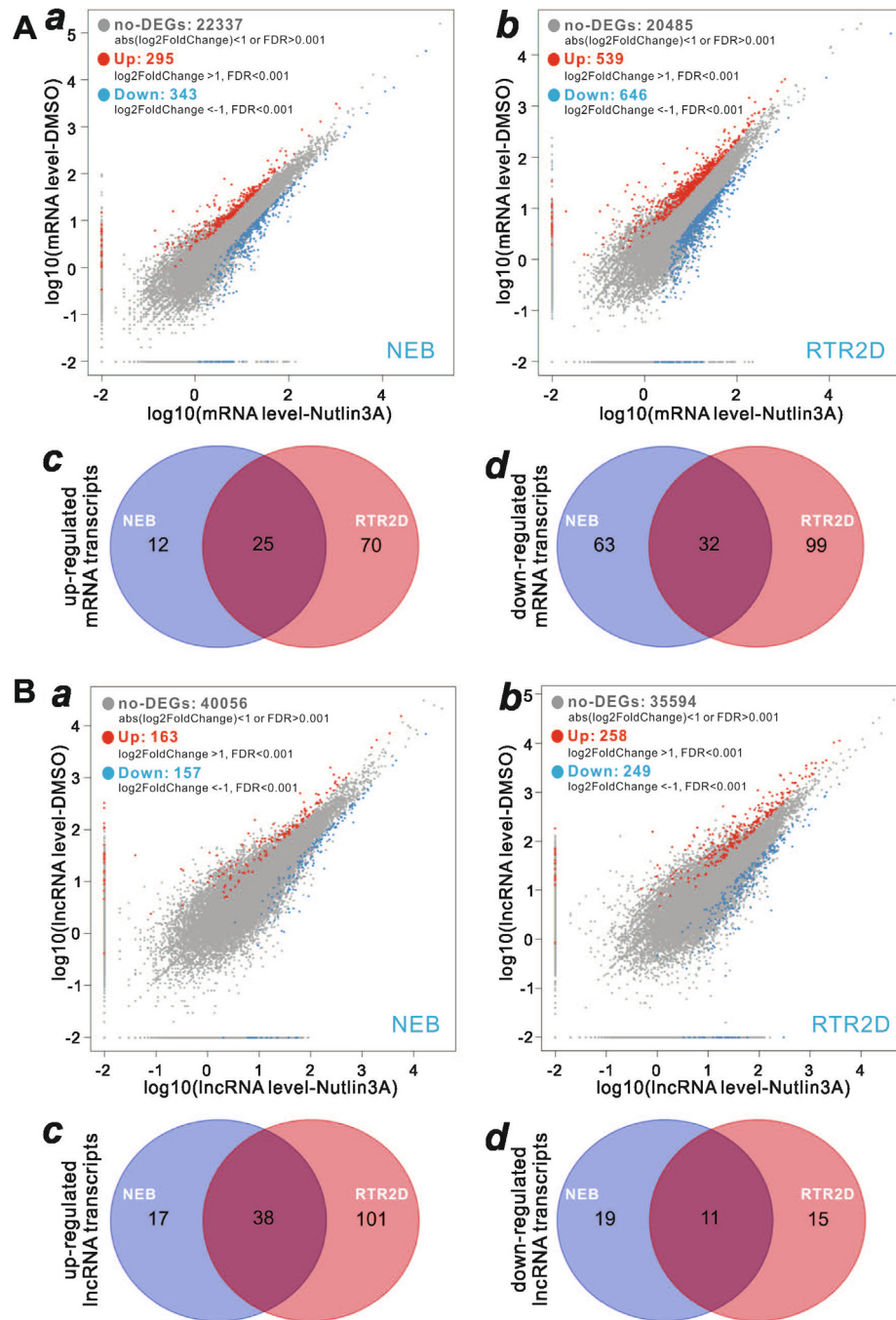
An ideal method for rRNA removal should be simplistic, efficient, reliable and yet cost-effective so it can be easily adapted for homemade RNA-seq library construction. Here, we developed the novel RTR2D reverse-transcriptase-mediated rRNA depletion methodology. We demonstrated that the RTR2D method was simple and efficient, and it depleted human or mouse rRNAs with high specificity without affecting mRNA and non-rRNA noncoding RNA transcripts. RNA-seq data analysis indicated that the RTR2D method yielded highly correlative transcriptomic landscape with



**Fig. 7. Transcriptomic comparison of the RNA-seq libraries prepared with the RTR2D and the NEBNext rRNA Depletion protocols.** Human total RNA (1.0  $\mu$ g) was subjected to the rRNA removal process by using the RTR2D procedure (R2D) and the NEBNext rRNA Depletion kit (NEB). The rRNA-depleted samples were used for RNA-seq library preparations using the Illumina protocol and subjected to NGS analysis (in replicate). (A) The average valid reads (RPKM) for the RTR2D (a) and the NEB kit (b) are depicted in various categories of transcripts. (B) Scatter plots and correlations of mRNA transcripts between the RTR2D and the NEB protocols in two different batches of preparations (a & b). (C) Scatter plots and correlations of lncRNA transcripts between the RTR2D and the NEB protocols in two different batches of preparations (a & b).

that of the NEBNext rRNA Depletion Kit at both mRNA and lncRNA levels. In a proof-of-principle study of determining the transcriptomic response to MDM2 inhibitor in human osteosarcoma cells, we found that the RNA-seq dataset from the RTR2D-depleted rRNA samples identified more differentially expressed mRNA and lncRNA transcripts than that from the NEBNext rRNA Depletion

samples, even though the overall transcriptome landscapes were similar and highly correlative, suggesting that RTR2D may have lower off-target depletion of non-rRNA transcripts. Thus, the reported RTR2D method should represent a novel, simplified and cost-effective approach for efficient rRNA removal for homemade RNA-seq library preparations.



**Fig. 8.** Nutlin3A-induced transcriptomic changes determined by RNA-seq analysis of the NGS libraries prepared with the RTR2D and the NEBNext rRNA Depletion protocols. Exponentially growing human osteosarcoma line SJS1 cells were treated with 2  $\mu$ M Nutlin3A or DMSO control for 24 h and subjected to total RNA isolation. Human total RNA (1.0  $\mu$ g) was subjected to rRNA removal by using the RTR2D procedure (R2D) and the NEBNext rRNA Depletion kit (NEB). The rRNA-depleted samples were used for RNA-seq library preparations using the Illumina protocol and subjected to NGS analysis. (A) Scatter plots of mRNA transcripts affected by DMSO (Y-axis) and Nutlin3A (X-axis) identified in the RNA-seq libraries prepared with the NEB protocol (a) or the RTR2D protocol (b). Venn diagrams were generated by using more stringent criteria for the differentially expressed transcripts for up-regulated (c) (total relative reads > 54) and down-regulated transcripts (d) (total relative reads > 13). (B) Scatter plots of lncRNA transcripts affected by DMSO (Y-axis) and Nutlin3A (X-axis) identified in the RNA-seq libraries prepared with the NEB protocol (a) or the RTR2D protocol (b). Significant up and down-regulated transcripts were defined as log2FoldChange > 1 and < -1, respectively, with a false detection rate (FDR) < 0.001. Venn diagrams were generated by using more stringent criteria for the differentially expressed transcripts for up-regulated (c) (total relative reads > 540) and down-regulated transcripts (d) (total relative reads > 20).

## Conclusion

In order to facilitate homemade RNA-seq library constructions, we developed a novel RTR2D methodology, and demonstrated that the RTR2D method was highly simplified and depleted human and mouse rRNAs with high specificity and efficiency. RNA-seq data

analysis indicated that RTR2D yielded highly correlative transcriptomic landscape with that of the commonly-used NEBNext rRNA Depletion Kit. In a proof-of-principle study of determining the transcriptomic response to MDM2 inhibitor in human osteosarcoma cells, we found that the RNA-seq dataset from the RTR2D-depleted rRNA samples identified more differentially expressed



mRNA and lncRNA transcripts than that from the NEBNext rRNA Depletion samples. These results suggest that RTR2D may have lower off-target depletion. Therefore, the RTR2D should be a valuable tool to deplete rRNAs for RNA-seq library constructions.

### Compliance with ethics requirements:

The authors declare that they have complied with all ethics requirements.

### Declaration of Competing Interest

The authors declare that they do not have any competing conflicts of interest.

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### Author contributions

T.C.H., Z.Z., A.H., R.C.H., H.H.L., M.J.L., and J.M.W. conceived and designed the study. Z.Z., B.H., X.W., J.F. and B.Z. performed the experiments and collected data. L.Y., Y.F., X.W., H.L., J.Z., M.Z., F. H., Y.M., M.P., W.W., A.J.L., B.L., H.D., Y.Z., C.N., M.W., and X.Z. participated in rRNA depletion experiments, provided essential experimental materials and assisted in qPCR data analysis and interpretations. T.C.H., Z.Z., R.C.H., H.H.L., M.J.L., and J.M.W. drafted the manuscript. All authors read, reviewed, revised and approved the manuscript.

### Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jare.2019.12.005>.

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