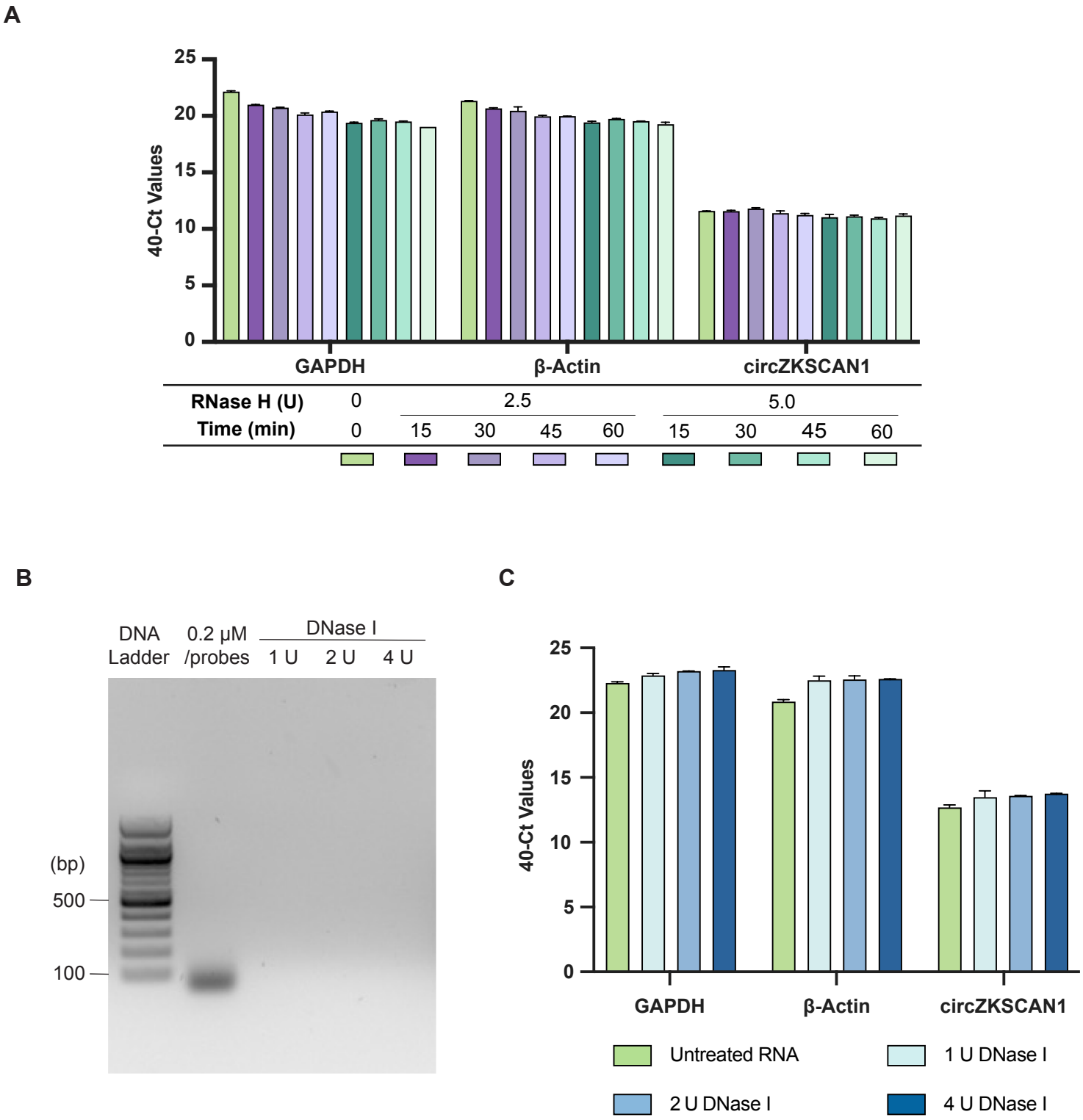
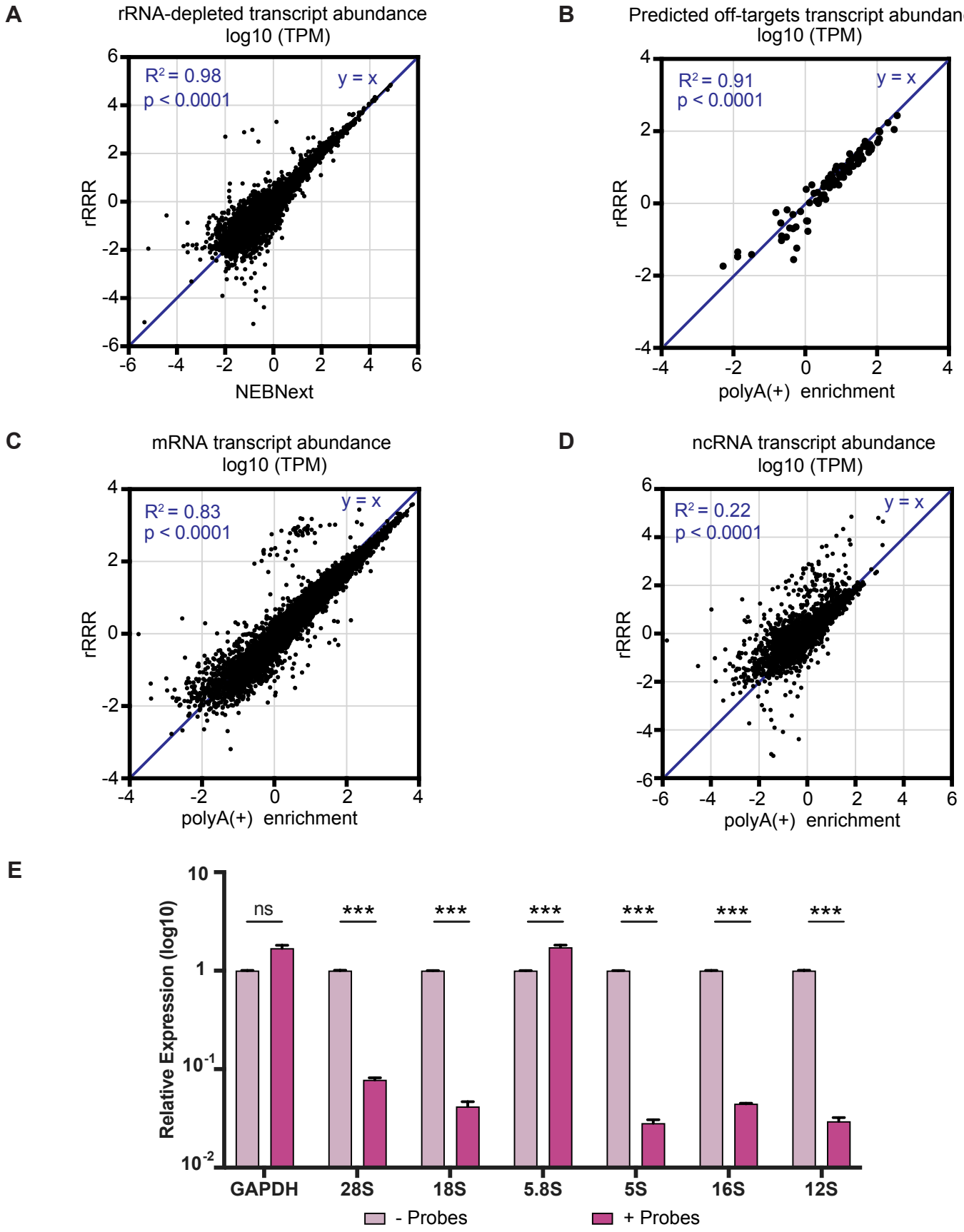


Supplemental Figure 1. Optimization of rRRR for removal of excess of probes and efficient rRNA depletion.

A) Total HeLa RNA was incubated with 0, 2.5, or 5.0 U of RNase H for 0, 15, 30, 45, or 60 minutes. RT-qPCR of the RNA after the incubations showed the effects of the enzyme on non-rRNAs (GAPDH, β-Actin, and circZKSCAN1) to determine an optimal condition that does not cause unwanted degradation of non-rRNA genes.

B) 0.2 μM probes were incubated with increasing units of DNase I to find optimum unit needed for removing the probes.

C) RT-qPCR of GAPDH, β-Actin, and cZKSCAN1 after treatment with 1 U, 2 U, or 4 U of DNase I (Mean of n = 3 technical replicates with SEM).



Supplemental Figure 2. rRRR in comparison to other commercial methods.

A) Analysis of rRNA-depleted RNA transcripts between rRRR and NEBNext treated RNA depicted $R^2 = 0.98$.

B) Predicted off-targets transcripts between rRRR treated RNA and polyA RNA had $R^2 = 0.91$.

C) Scatter plots for mRNA and (D) ncRNA between rRRR-treated RNA and polyA(+) RNA showed $R^2 = 0.83$ and 0.22 respectively. RNA-seq was performed in duplicate. TPM: Transcripts Per Million, R^2: Coefficient of determination.

E) Relative expression of transcripts in "- Probes" and "+ Probes" from HeLa RNA depleted for rRNAs using RiboMinus Kit. Representative data is shown as mean of n = 3 biological replicates with SEM.
ns: $p > 0.05$, * : $p \leq 0.05$, ** : $p \leq 0.01$, *** : $p \leq 0.001$ (unpaired t-test).

A. Probes Hybridization

RNA Amount	Volume (µL)	46.6 uM Probes (µL)	1M Tris-Cl (pH 7.4) (µL)	5M NaCl (µL)	H_2O
1 µg	x	2.0	1.0	0.4	up to 10 µL
10 µg	x	20.0	3.0	1.2	up to 30 µL
20 µg	x	40.0	6.0	2.4	up to 60 µL

Incubation: 95 ˚C for 2 minutes, TD from 95 ˚C to 22 ˚C 0.1C/sec, and hold at 22 ˚C for 5 minutes

B. RNase H Reaction

RNA Amount	Vol. From Previous Reaction (µL)	RNase H (µL)	10X RNase H Buffer (µL)	H_2O
1 µg	10.0	0.5	1.5	up to 15 µL
10 µg	30.0	5.0	4.0	up to 40 µL
20 µg	60.0	10.0	8.0	up to 80 µL

Incubation: 37 ˚C for 30 minutes

C. DNase I Reaction

RNA Amount	Vol. From Previous Reaction (µL)	DNase I (µL)	10X DNase I Buffer (µL)	H_2O
1 µg	15.0	1.0	2.0	up to 20 µL
10 µg	40.0	10.0	6.0	up to 60 µL
20 µg	80.0	20.0	12.0	up to 120 µL

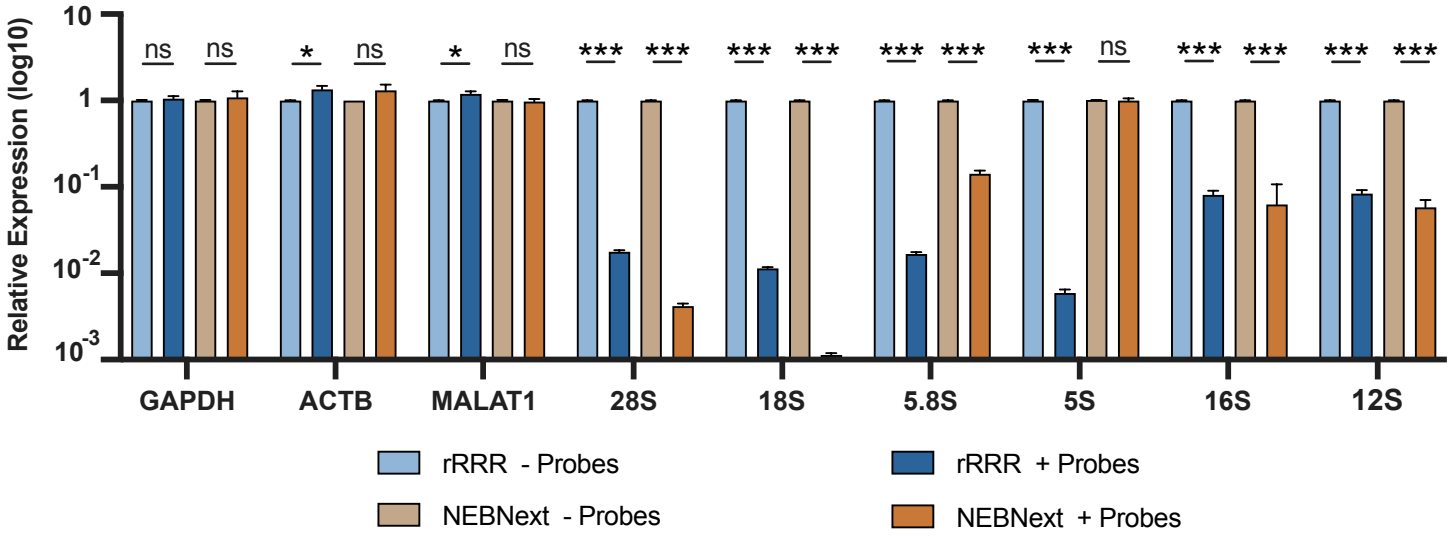
Incubation: 37 ˚C for 30 minutes

D. Bead Purification

RNA Amount	Volume of Previous Reaction	Beads (µL)
1 µg	20.0	36.0
10 µg	60.0	108.0
20 µg	110.0	198.0

Followed manufacturer's protocol

Supplemental Figure 3. Reactions volumes used for rRNA depletions with 1 µg, 10 µg, and 20 µg of starting input RNA.



Supplemental Figure 4. Relative expression of transcripts in "- Probes" and "+ Probes" extracted from human lymph node FFPE cores was subjected to rRNA depletion using rRRR and NEBNext.

Representative data is shown as mean of n = 3 biological replicates with SEM.
ns: $p > 0.05$, * : $p \leq 0.05$, ** : $p \leq 0.01$, *** : $p \leq 0.001$ (unpaired t-test).

A

Procedure	Reagent	Catalog #	µL per ug reaction	Cost per 1 µg Reaction
Baldwin et. al.	25 nmole DNA Oligo 50-mer (n = 195 probes), 100 µM	Sigma	3.3	$0.24
	Lucigen Thermostable RNase H, 5 U/µl	H39500	2.0	$8.84
	Turbo DNase, 2 U/µl	AM2238	7.5	$2.28
	Beckman Coulter Agencourt RNAClean XP Beads	A63987	176.0	$4.00
			Total Cost	**$15.40**

B

Procedure	Reagent	Catalog #	µL per ug reaction	Cost per 1 µg Reaction
RTR2D	25 nmole DNA Oligo 35-mer (n = 30 probes), 50 µM	Sigma	6.0	$0.15
	NEB M-MuLV Reverse Transcriptase, 200 U/µl	M0253S	1.0	$1.74
	NEB RNase Inhibitor, 2 U/µl	M0314S	2.0	$0.45
	NEB Exonuclease I, 2 U/µl	M0293S	2.0	$2.16
	NEB RNase H, 2 U/µl	M0297S	2.0	$1.05
	NEB DNase I, 2 U/µl	M0303S	2.0	$3.28
	NEB dNTPs, 10 mM	N0447S	2.0	$0.31
	Thermo Phenol Chloroform based purifications	15593031	600	$1.00
			Total Cost	**$10.14**

Supplemental Figure 5. Cost breakdown of other non-commercial RNase-H based rRNA depletion methods

A) Cost of reagents for RNase H based rRNA depletion method by Baldwin et. al. (9) was calculated. The cost for depleting rRNAs from 1 µg of total RNA amounted to $15.4.

B) Cost calculations of RTR2D (10), totaled to $10.14 for depleting rRNAs from 1 µg of total RNA.