Supplemental Methods

DNA template sequences

negative control construct

GCGGCCGCctgtcactttggaaaaaaaatatacgctaagatttttggagaatagcttaaattgaagtttt
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tttggtggattactagctaatagcaatctatttcaaagaattcaaacttgggggaatgccttgttgaata
gccggtcgcaagactgtgattcttcaagtgtaacctcctctcaaatc**CGTGGCGCGGACTCGAGGAACGG
ACGCGAAGCTAACGGAATAGTGACTCTTC**agcgatatcaaacgtaccattccgtgaaacaccggggtatc
tgtttggtggaacctgattagaggaaactcaaagagtgctatggtatggtgacggagtg***GGCGCC*****CTCGA
G**

spy terminator construct

GCGGCCGCctgtcactttggaaaaaaaatatacgctaagatttttggagaatagcttaaattgaagtttt
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TCAAcgtaccattccgtgaaacaccggggtatctgtttggtggaacctgattagaggaaactcaaagagt
gctatggtatggtgacggagtg**GGCGCC*****CTCGAG***

spy terminator construct, T to C mutant

GCGGCCGCctgtcactttggaaaaaaaatatacgctaagatttttggagaatagcttaaattgaagtttt
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AAGACCGCCGGTCTTGTCCACTACCTTGCAGTAATGCGGTGGACAGGATCGGCGGCCCCTCCCCTCTCCT
CCAAcgtaccattccgtgaaacaccggggtatctgtttggtggaacctgattagaggaaactcaaagagt
gctatggtatggtgacggagtg**GGCGCC*****CTCGAG***

spy terminator construct, short stem loop mutant

GCGGCCGCctgtcactttggaaaaaaaatatacgctaagatttttggagaatagcttaaattgaagtttt
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ggtatctgtttggtggaacctgattagaggaaactcaaagagtgctatggtatggtgacggagtg**GGCGC
C*****CTCGAG***

rDNA sequence construct

GCGGCCGCctgtcactttggaaaaaaaatatacgctaagatttttggagaatagcttaaattgaagtttt
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GGAGTTATCTTTTCTTCTTAACAGCTTATCACCCcgtaccattccgtgaaacaccggggtatctgtttgg
tggaacctgattagaggaaactcaaagagtgctatggtatggtgacggagtg**GGCGCC*CTCGAG***

rDNA sequence construct, T to C mutant

GCGGCCGCctgtcactttggaaaaaaaatatacgctaagatttttggagaatagcttaaattgaagtttt
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TCCGGAACCTGGATATG*G*ATTCTTCACGGTAACGTAACTGAATGTGGAGACGTCGGCGCGAGCCCTGGGA
GGAGCCACTCCCCTCCTCCAACAGCTTATCACCCcgtaccattccgtgaaacaccggggtatctgtttgg
tggaacctgattagaggaaactcaaagagtgctatggtatggtgacggagtg**GGCGCC*CTCGAG***

rDNA sequence construct, upstream scramble mutant

GCGGCCGCctgtcactttggaaaaaaaatatacgctaagatttttggagaatagcttaaattgaagtttt
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tttgtaatgggaggggggtttagtcatggagtacaagtgtgaggaaaagtagttgggaggtacttcatg
GgaaagGagttgaagaGaagttGgaaaagagtttggaaaGgaattGgagtaggcttgtcgttcgttatgt
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TCCGGAACCTGGATATG*G*ATTCTTCACGGTAACGTAACTGAATGCCGTAGTGCTAAGGTAACCTACAACG
TGCTTTATCTTTTCTTCTTAACAGCTTATCACCCcgtaccattccgtgaaacaccggggtatctgtttgg
tggaacctgattagaggaaactcaaagagtgctatggtatggtgacggagtg**GGCGCC*CTCGAG***

Cell culture and immunoprecipitation

Per replicate, three litres of *S. cerevisiae* bearing an RPA135 C-terminal (3x-HA 7x- his) tagged mutant (*MATα ade2-1 ura3-1 trp1-1 leu2-3 112 his3-11,15 can1-100 RPA135-(HA)3-(His)7:TRP1Mx6 rpa190Δ::HIS3Mx6* carrying *pRS315-RPA190*) [1] were grown in YEPD media to A_{600} = 0.3 at 30°C with nutation. The cells were collected via filtration, then frozen in liquid nitrogen. The cells were then cryogenically lysed via ten one-minute cycles of 16mm amplitude grinding in the Mikro-Dismembrator II grinding mill. The grinding cup was pre-chilled by immersion in liquid nitrogen, and the re-immersed for 1 minute in between grinding cycles.

Grindates for each liter were dissolved in five-fold weight amounts of ice-cold lysis buffer (20 mM Tris Cl pH 7.9, 0.4% Triton X-100, 0.1% NP-40, 100 mM NH_4Cl, 5 mM EDTA Na pH 8.5, 1x HALT Protease Inhibitor, 25 U/ml SUPERase-in RNAse Inhibitor). Aliquots of Pierce Anti-HA magnetic beads equal to 4% of lysis buffer volume were washed 4x with ice-cold lysis buffer. The lysates were centrifuged at 16,000 x g for 15 minutes. The lysate supernatants were combined with pre-washed aliquots of Pierce Anti-HA magnetic beads and set to nutation at 4°C

for 3 hours. The beads were then isolated via magnet and washed 4x with ice-cold wash buffer (20 mM Tris Cl pH 7.9, 0.4% Triton X-100, 0.1% NP-40, 300 mM KCl, 50 mM EDTA Na pH 8.5, 25 U/ml SUPERase-in RNAse Inhibitor). Finally, the beads were resuspended in 900 µl TES (10 mM Tris Cl pH 7.5, 1% SDS) and extracted 3x with 900 µl aliquots of acidic (pH 4.3) phenol, then 2x with 900 µl aliquots of chloroform. The remaining aqueous solutions were combined with 1.2 ml ammonium acetate precipitation solution (1M ammonium acetate, 95% ethanol) and 2 µl glycoblue, and set to -80°C for at least 2 hours. The solutions were centrifuged at 16,000 x g for 1 hour at 4°C. The pellets were then washed 2x with 750 µl 75% ethanol, and resuspended in 10 µl 10 mM tris Cl pH 7.0.

Linker ligation, zinc chloride fragmentation, and size selection

The isolated RNA samples from each liter were denatured at 80°C for 2 minutes, then put on ice. A 5' adenylated DNA linker end-blocked with a 3' di-deoxy C (5'-/5rApp/CTCCACGAGTCATCCGC/3ddc/-3', Integrated DNA Technologies) was denatured at 80°C for 3 minutes, then combined with RNA samples and buffering conditions for a labelling reaction with the following concentrations (12% PEG MW 8000, 1x T4 RNA Ligase buffer (NEB), 5 µM linker, and 10 U / µl T4 RNA Ligase 2, truncated (NEB)). The reactions were set to 25°C for three hours. In order to fragment the RNAs 2.2 µl zinc chloride fragmentation buffer (100 mM tris Cl ph 7.0, 100 mM $ZnCl_2$) was added to each ligation reaction and set to 70°C for 20 minutes. The fragmentation reactions were then quenched by that addition of 2.5 µl 200 mM EDTA Na pH 8.5, 2 µl glycoblue, and 1 ml ammonium acetate precipitation solution, and set to -80°C for at least 2 hours. The solutions were centrifuged at 16,000 x g for 1 hour at 4°C. The RNA pellets were then washed 2x with 750 µl 75% ethanol. The RNA samples were denatured at 98°C for 5 minutes, loaded on a pre-run 10% polyacrylamide gel, and run at 700 volts for 70 minutes. The gel was stained for 30 minutes at room temperature in 1X SYBR Gold stain in 1X TBE buffer. The 30 to 330 nt regions were excised for each replicate. The gel slices were pulverized and combined with 600 µl H_2O, and set to -80°C for 14 hours. The gel slurries were then set to 70°C for 20 minutes, and the liquid was isolated via a 5 minute 16,000 x g centrifugation in Costar Spin-X Centrifuge Tube Filters (Corning). The solutions were then combined with 37.5 µl 3M ammonium acetate, 2 µl Glycoblue, and 1.125 ml isopropanol. The solutions were set to -80°C for 2 hours, then centrifuged at 16,000 x g for 1 hour at 4°C. The RNA pellets were washed twice with 750 µl 75% ethanol, and dried at room tempurature for 25 minutes. The pellets were then resuspended in 10 µl 10 mM Tris Cl pH 6.9.

Reverse Transcription

The size-selected RNA samples were combined with a 5' phosphorylated reverse transcription primer (RT primer) with two internal 18 atom hexa-ethyleneglycol spacers (5'-/5Phos/CTGTAGGCACCATCAATG ATCGTCGGA/isp18/CACTCA/isp18/CGTCTCTTCTGCGGATGACTCGTGGAG-3', Integrated DNA Technologies) with buffering conditions (1X 5X First Strand Buffer, 400 µM each dATP, dCTP, dGTP, dTTP, and 250 nM RT primer), denatured at 65°C for 5 minutes, and set on ice. The following components were then added to complete the full reverse transcription reaction (5 U/µl SUPERase-in RNAse Inhibitor, 4 mM DTT, and 8 U/ µl Superscript III (Invitrogen). The solutions were set to 50°C for 30 minutes. In order to get rid of the residual RNA 1.8 µl 1M NaOH was added to each reaction and set to 98°C for 20 minutes. The 70 to 330 nt range cDNAs for each sample were size-selected and precipitated as described above, except using

3M sodium for precipitation instead of 3M sodium acetate, and resuspended in 15 µl 10 mM Tris Cl pH 7.0.

Circularization, PCR Amplification, Size Selection, and Library Desalting

Circularization with CircLigase II ssDNA Ligase (Epicentre) was performed per manufacturer's instructions, with the addition of a second 1 µl aliquot of Circligase and 60-minute incubation step at 60°C following the first. The Circularized cDNAs were then amplified in a PCR reaction with the following concentrations: (1x Phusion Buffer, 94 nM forward primer, 94 nM reverse primer, 330 µM each dATP, dCTP, dGTP, dTTP, and 0.019 U/µl phusion polymerase (NEB)). Each sample was amplified with a unique combination of forward and reverse primers to allow for demultiplexing (table 1.) The reactions amplified by the following PCR cycle: 98°C for 30 seconds, 12x (98°C for 10 seconds, 60°C for 10 seconds, 72°C for 30 seconds.) The 160-330 nt range of library DNAs for each sample were size-selected and precipitated as described above, and resuspended in 25 µl 10 mM tris acetate pH 8.0. Libraries were desalted using illustra Microspin S-200 HR Columns (GE) according to manufacturer's instructions.

Supplementary Table 1. Library Amplification Primers for NETSeq

Replicate	reverse primer sequence	forward primer sequence
1st	AATGATACGGCGACCACCGAGATCTACACtagatcgcCGTCTCTTCTGCGGATGACTCG	CAAGCAGAAGACGGCATACGAGATtcgccttaTCCGACGATCATTGATGGTGCC
2nd	AATGATACGGCGACCACCGAGATCTACACtagatcgcCGTCTCTTCTGCGGATGACTCG	CAAGCAGAAGACGGCATACGAGATctagtacgTCCGACGATCATTGATGGTGCC
3rd	AATGATACGGCGACCACCGAGATCTACACtagatcgcCGTCTCTTCTGCGGATGACTCG	CAAGCAGAAGACGGCATACGAGATttctgcctTCCGACGATCATTGATGGTGCC

Sequencing

Libraries were sequenced on the NextSeq500 according to manufacturer's instructions, using the following index primer (5'-CCATCAATGATCGTCGGA-3', Integrated DNA Technologies) and sequencing primer (5'-CGTCTCTTCTGCGGATGACTCGTGG-3', Integrated DNA Technologies.) The image **b**ase**c**a**l**l (bcl) files from the Illumina NextSeq500 were converted to fastq file format using Illumina's bcl2fastq software (ver. 2.18.0.12.)

Analysis software

Sequence trimming, alignment, and data formatting were performed with Trim Galore (ver. 0.4.1,) Burrows-Wheeler Aligner (BWA) (ver. 0.6)[2], Samtools (ver. 1.3.1)[3], and Bedtools (ver. 2.9.1)[4]. Subsequent analysis was performed in R (ver. 3.3.1)[5] using the following packages: ggplot2 (ver. 2.2.1)[6], dplyr (ver. 0.7.4)[7], and ggseqlogo (ver 0.1)[8]. Unix and R analysis scripts available upon request.

Sequence Trimming and Alignment

All sequences were trimmed of the first two bases (AG in all sequences by library design.) Where detected, the 3' library sequence (CTGTAGGCACCAT) and all subsequent bases were also removed. The trimmed sequences were then aligned to the *S. cerevisiae* genome (SacCer3 assembly, downloaded 25 October 2017, URL: http://hgdownload.cse.ucsc.edu/goldenPath/sacCer3/bigZips/chromFa.tar.gz) via the BWA-MEM tool, with a minimum alignment score threshold of 20.

Sequence Logo Generation

127 positions were “significant” in all three replicates. To represent the RNA:DNA hybrid a 14 nt sequence (comprised of the 9 bases upstream of the “significant” position, the “significant” position itself, and the 4 nucleotides downstream) was isolated for each of these positions. These sequences were then used to produce a sequence logo via ggseqlogo using default settings.

Supplemental Figures

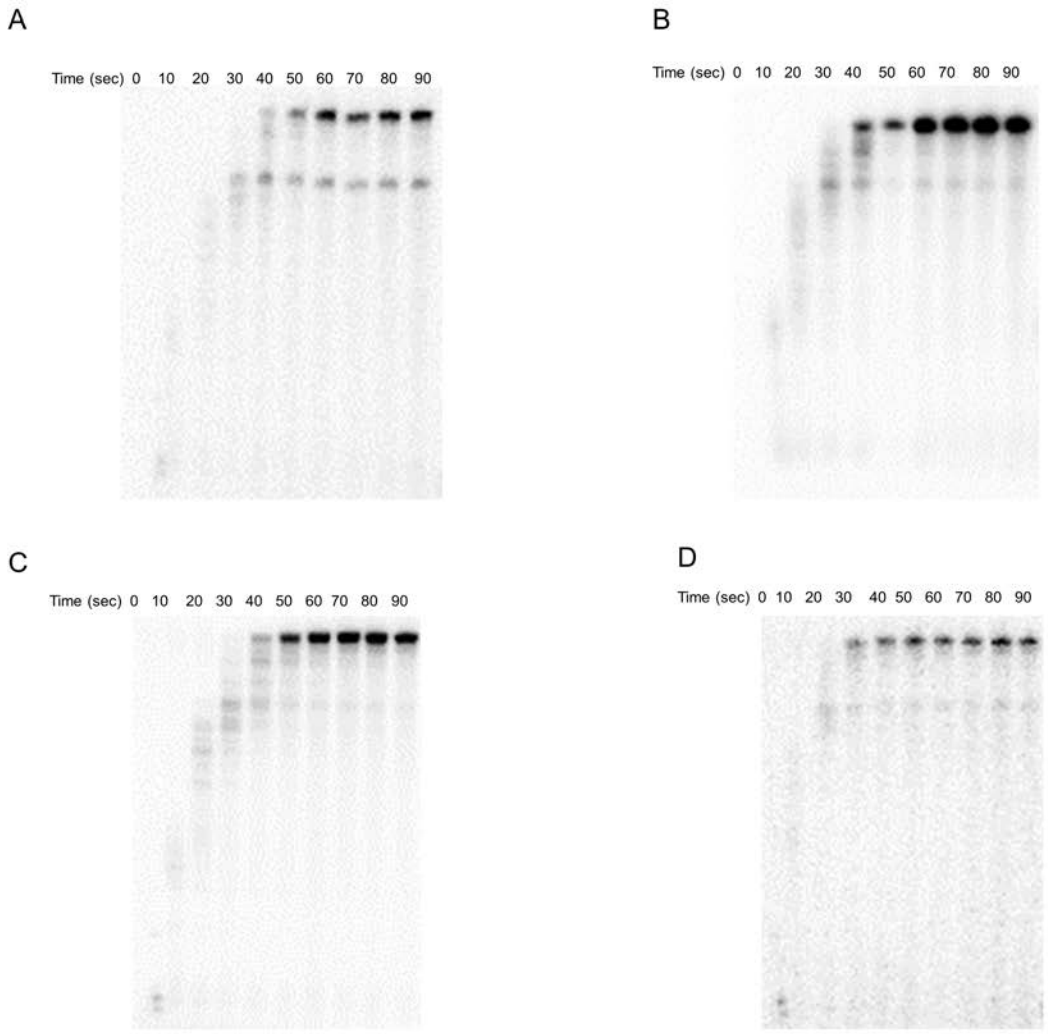

Figure S1. S1a) Representative polyacrylamide gel for Fig 2c. S1b) representative polyacrylamide gel for Fig 3b. S1c) Representative polyacrylamide gel for Fig 3C. S1d) Representative polyacrylamide gel for Fig 5a, 100 µM UTP.

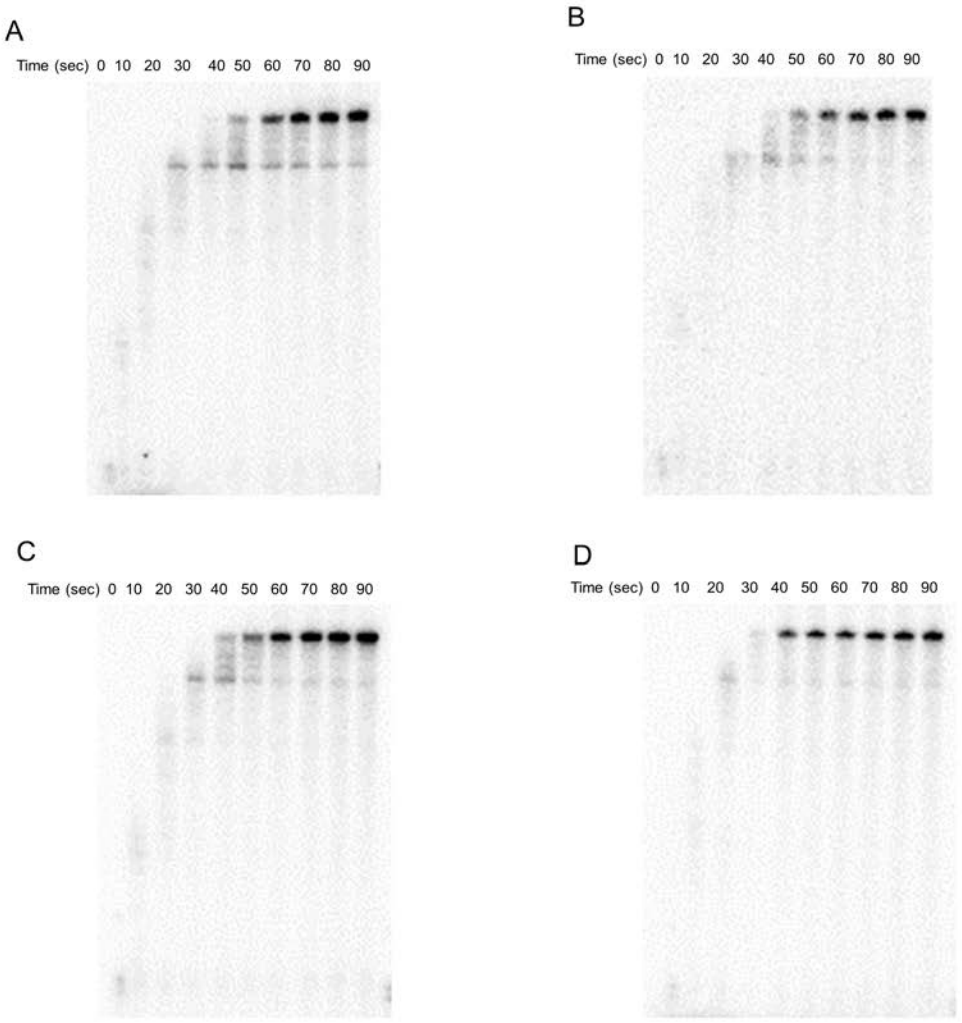

Figure S2. S2a) Representative polyacrylamide gel for Fig 4a. S2b) representative polyacrylamide gel for Fig 4b. S2c) Representative polyacrylamide gel for Fig 4c. S2d) Representative polyacrylamide gel for Fig 5b, 100 µM UTP.

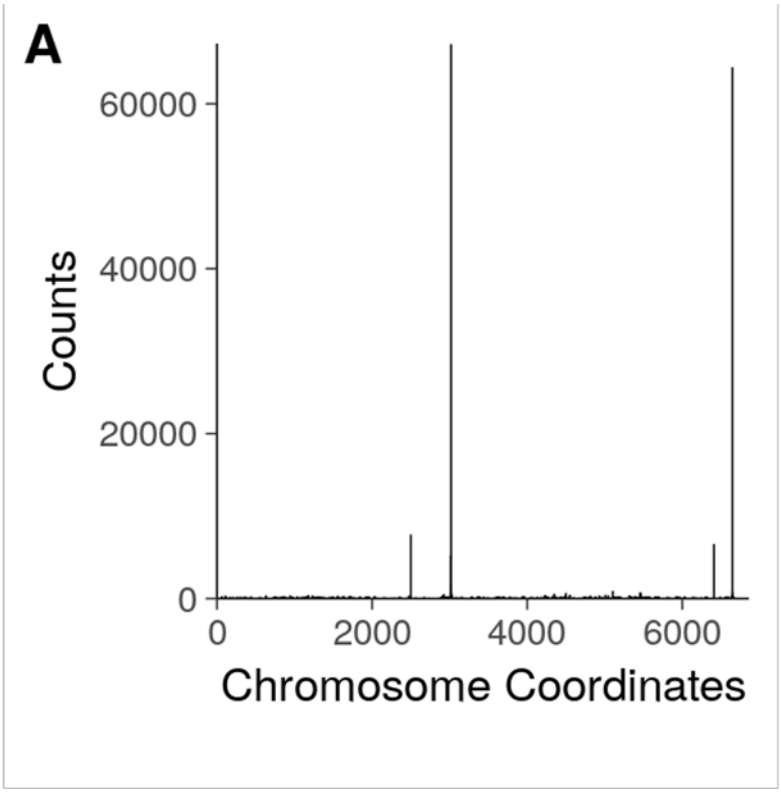

Figure S3. a) NETSeq Pol I 3’ end densities in the 35S gene including the ends of mature rRNA species.

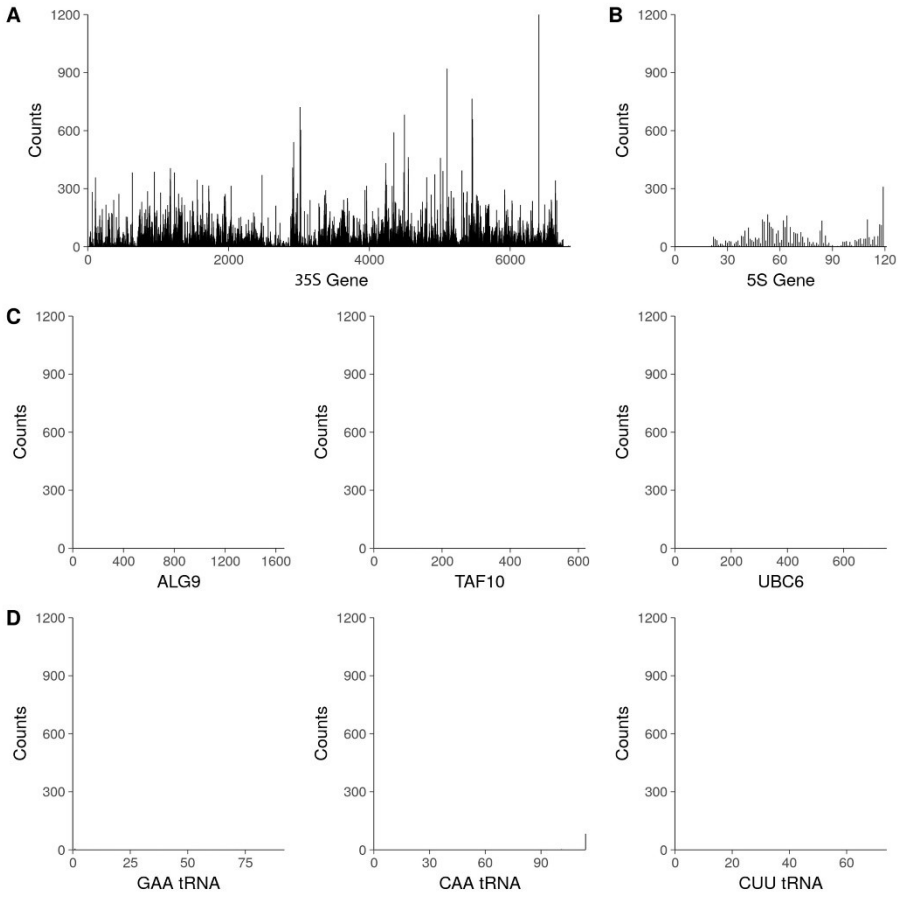

Figure S4. a) NETSeq 3’ end densities in the 35S gene. b) NETSeq 3’ end densities in the 5S gene. c) NETSeq 3’ end densities in three Pol II transcribed genes. d) NETSeq 3’ end densities in three Pol III transcribed genes.

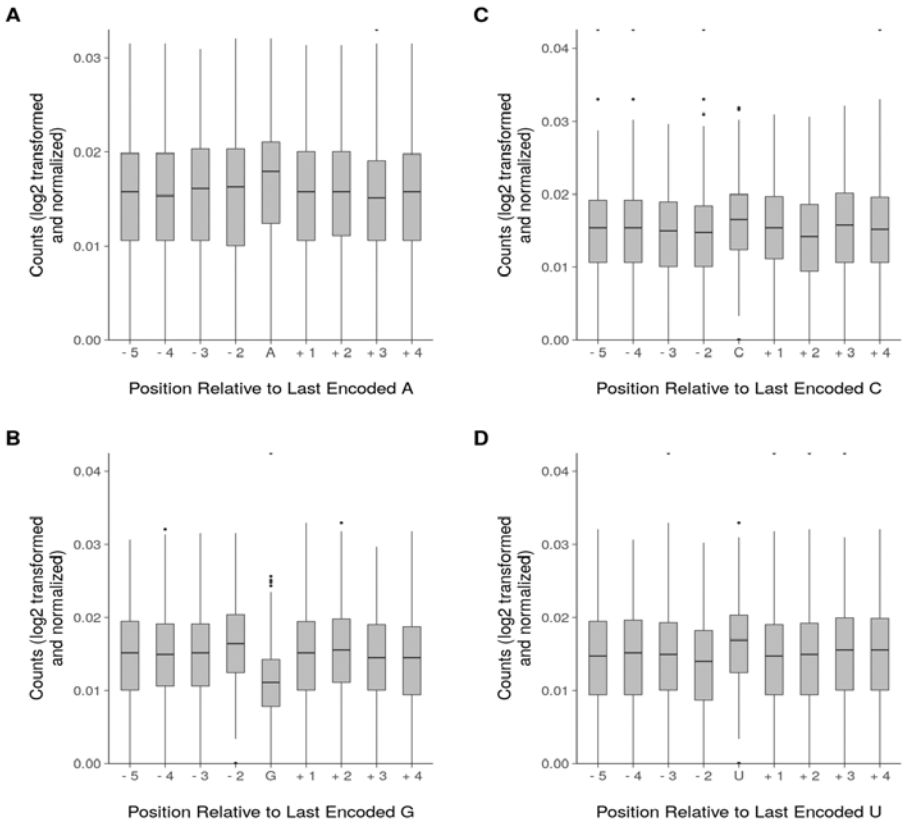

Fig S5. S5a-d) box plots of position occupancies proximal to last encoded A, C, G, and U residues, respectively. Counts are log2 transformed and normalized.

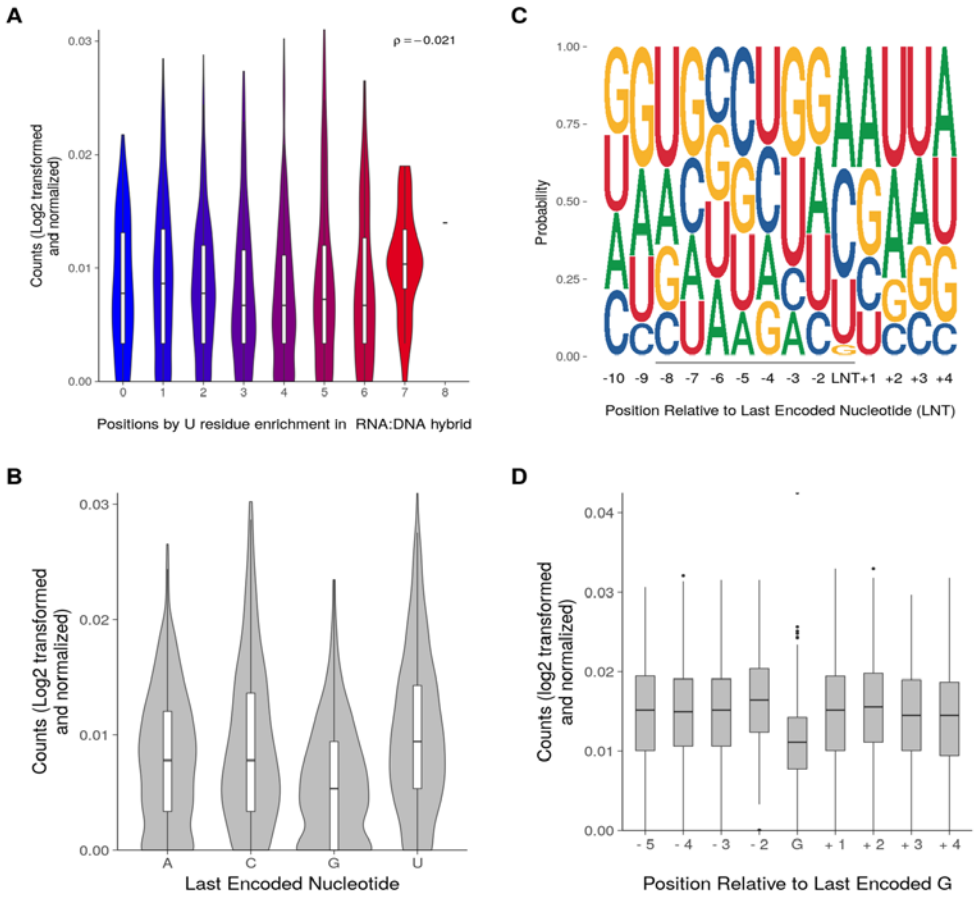

Figure S6. S6a) Violin plot of 3’ spacer positions sorted by RNA:DNA hybrid U content, with incorporated box plots. Counts are log2 transformed and normalized. Correlation coefficient determined by spearman correlation test, p-value < 1*10-8. S6b) Violin plot with incoporated box plots of rDNA spacer position occupancies sorted by last encoded nucleotide. Comparison by Kruskal Wallis test produced a p-value < 1*10-8. S6c) Sequence logo of the RNA:DNA hybrid corresponding to top 3’ spacer positions conserved in all three sets. S6d) box plot of spacer position occupancies proximal to last encoded G residues. Counts are log2 transformed and normalized. Comparisons by Mann-Whitney U test. ns = not significant, * = p-value < 5*10-6, *** = p-value < 5*10-16

Supplemental References

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3. Li H., B.H., A. Wysoker , T. Fennell, J. Ruan, N. Homer, G. Marth, G. Abecasis, R. Durbin, *The Sequence alignment/map (SAM) format and SAMtools. Bioinformatics.* Bioinformatics, 2009. **25**: p. 2078-2079.
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7. Hadley W., F.R., L. Henry, K. Müller, *dplyr: A Grammar of Data Manipulation.* 2017.
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