

Figure S1

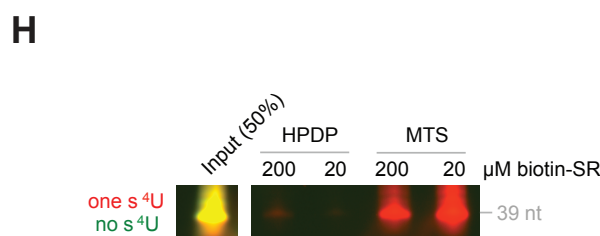
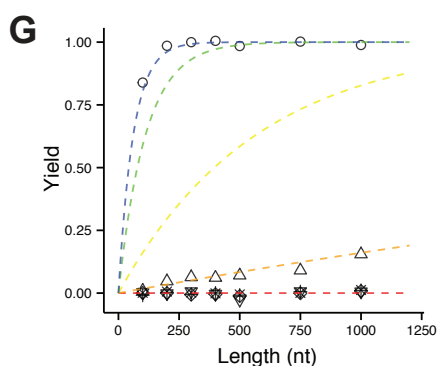
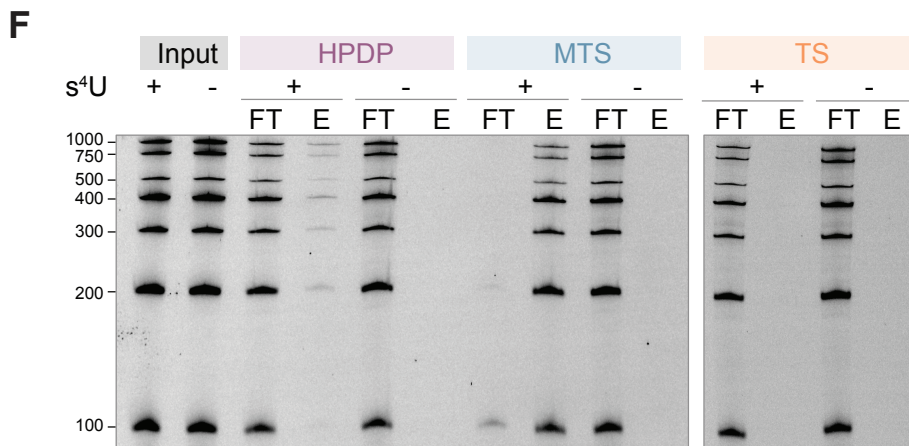
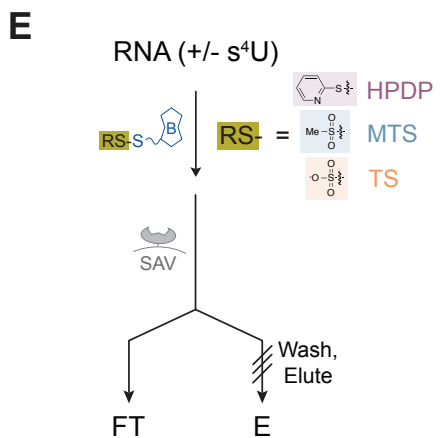
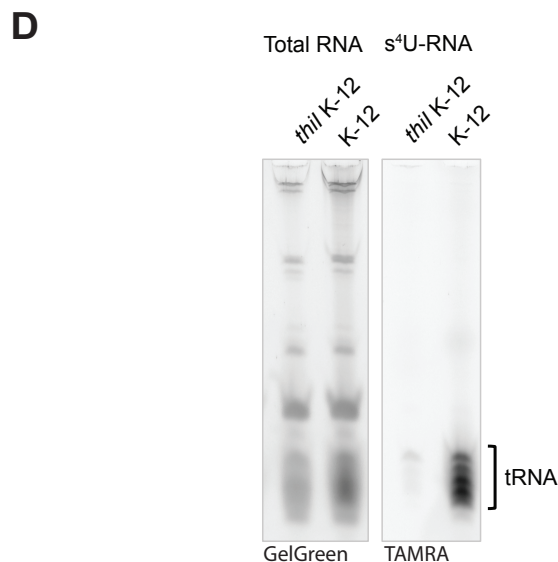
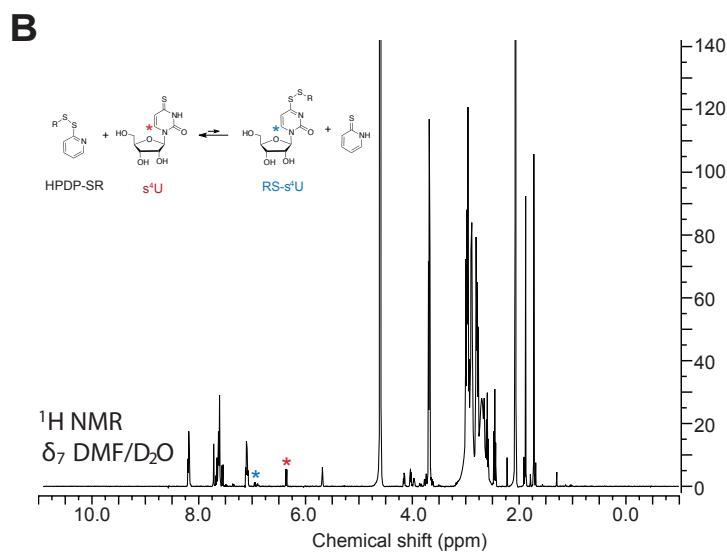
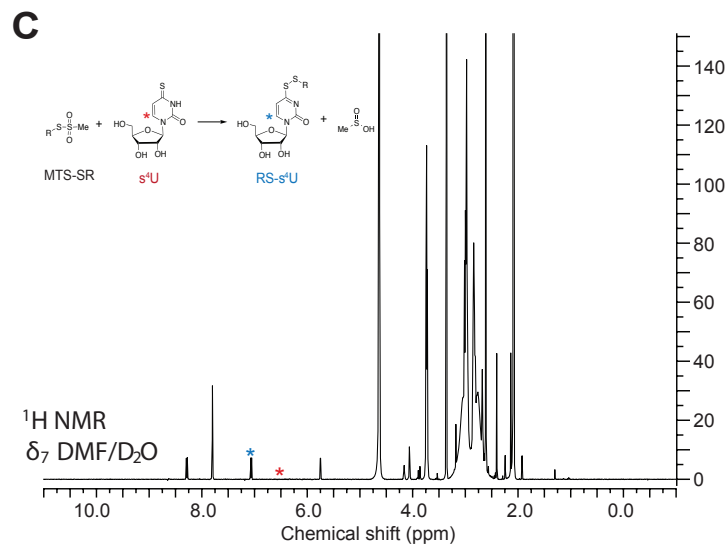
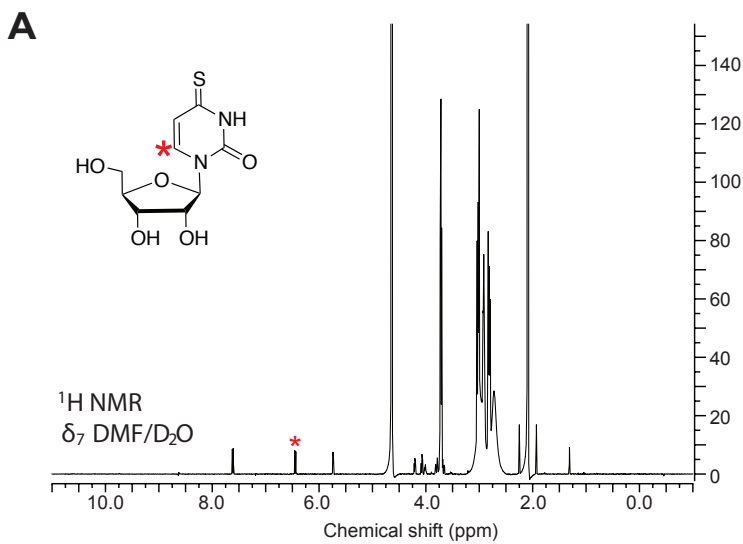
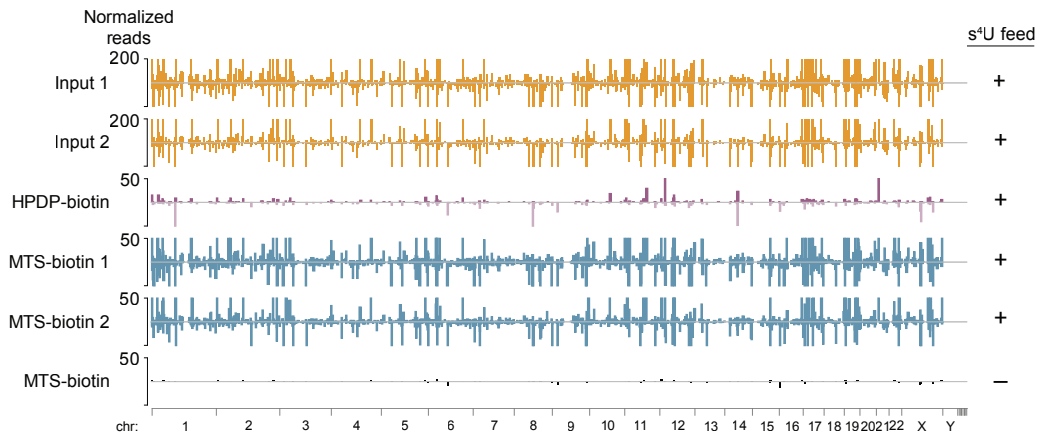
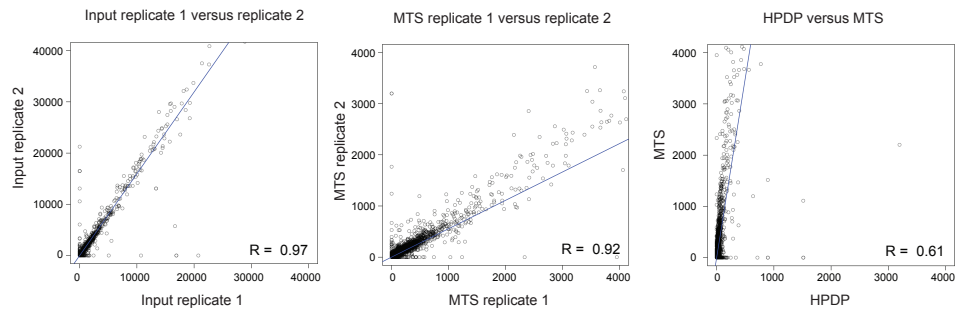


Figure S2

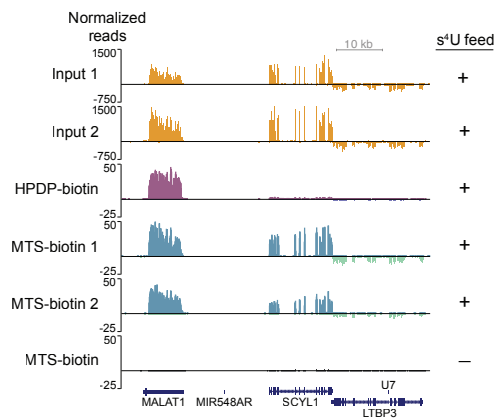
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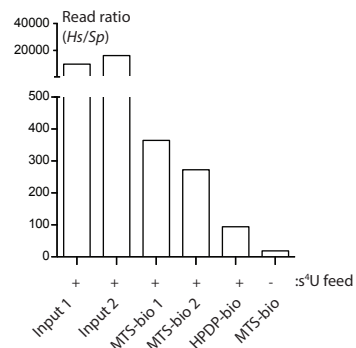
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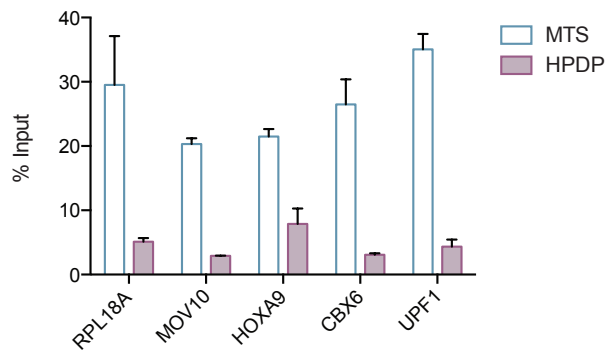
C



D



E



F

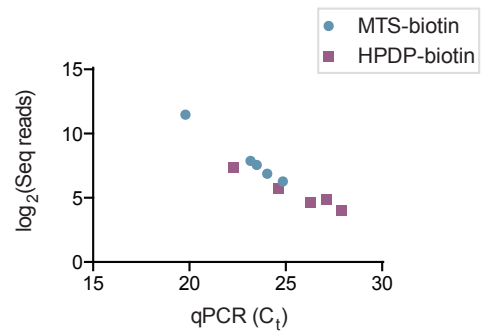
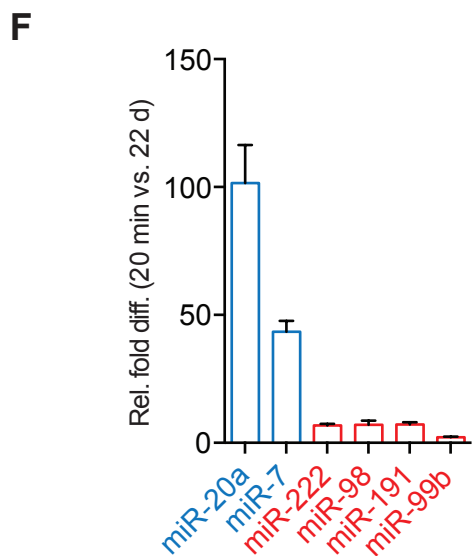
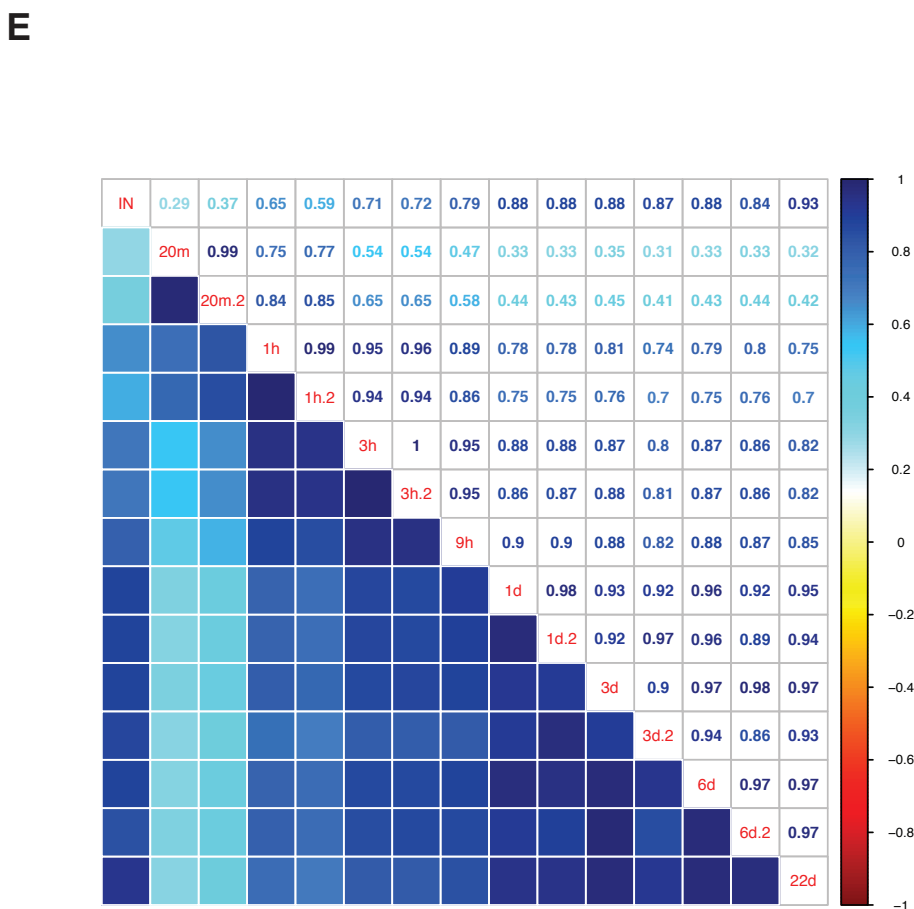
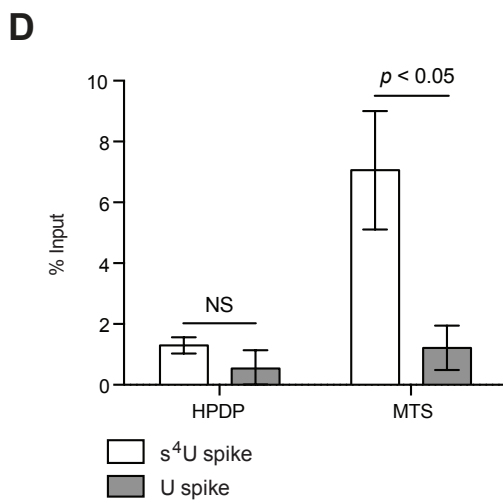
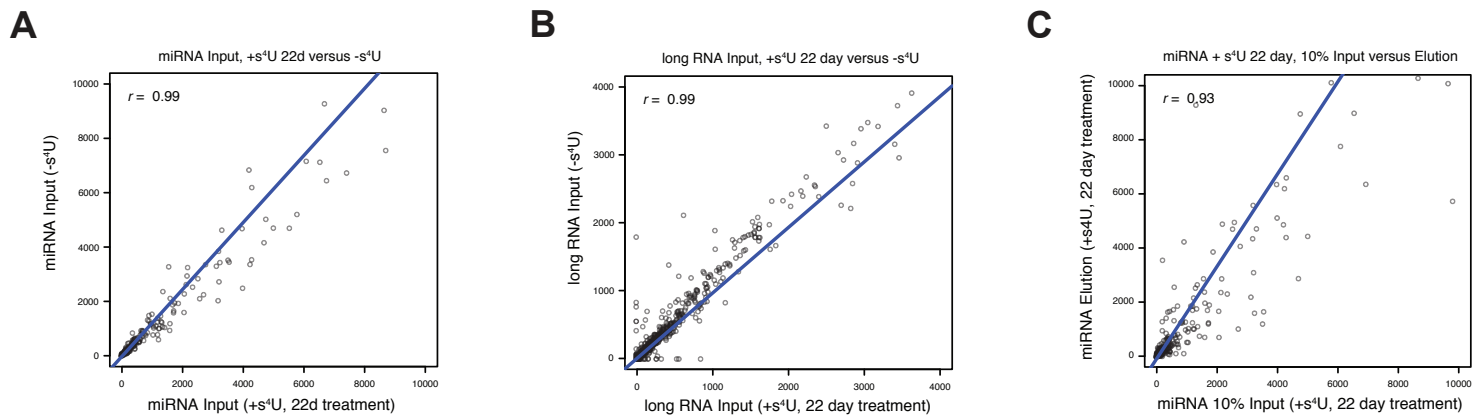


Figure S3



SUPPLEMENTAL FIGURE AND TABLE LEGENDS

Figure S1. Reactivity of activated disulfides with s^4U and *in vitro* modulation of bias in MTS- and HPDP-biotin enrichments, related to Figure 1

(A) 1H NMR spectrum of s^4U alone. Peak labeled with a red “*” corresponds to the starred proton in the s^4U structure. (B) 1H NMR spectrum of s^4U when treated with methane methylthiosulfonate (MeMTS), the same reactive disulfide of MTS-biotin. MeMTS was incubated with s^4U for 30 min and the extent of disulfide exchange was monitored by the chemical shift in proton labeled with a red “*”. Peak labeled with a blue “*” represents the chemical shift upon disulfide bond formation. (C) 1H NMR spectrum of s^4U when treated with a compound containing the same functional group of HPDP-biotin. 3-[2-Pyridyldithio]propionyl hydrazide (PDPH) was incubated with s^4U for 2 hr and the extent of disulfide exchange was monitored by changes in chemical shift as in (B). (D) RNA from *E. coli* K-12 cells was reacted with MTS-TAMRA fluorescent dye and visualized on a 5% urea-PAGE gel. K-12 cells express ThiI, an enzyme that selectively modifies U8 of tRNA to s^4U8 (Mueller et al., 1998b). RNA from a $\Delta thiI$ knockout shows little TAMRA signal (traces of unmethylated 2-thiouridine on tRNA can still react), whereas a strong TAMRA signal is present in the K-12 cells only in tRNA. Total RNA was stained with GelGreen. (E) Schematic of *in vitro* enrichment of s^4U -RNA using an RNA ladder. An RNA ladder was *in vitro* transcribed with Cy5-CTP and with or without added s^4UTP . s^4U -RNAs were enriched by reacting with disulfide-activated biotin derivatives using either HPDP, MTS, or thiosulfonate (TS, an alternative disulfide activated biotin reagent) chemistry. (F) Input, flow-through, and elution RNAs were analyzed by urea-PAGE and visualized by Cy5 fluorescence. Band intensities were quantified using ImageJ. (G) Comparison

between the yields observed in (E) and expected enrichment using models that assume different biotinylation efficiencies. In all cases modeled lines assume ratio of $s^4U/U_{total} = 0.075$ to determine the expected yield given different biotinylation efficiencies (y_{bio}) based on the equation:

$$\text{yield RNA} = \sum_{j=0}^{N_i} [1 - (1 - y_{bio})^j] p(U_i = j)$$

(see also the “Modeling expected yields” section of the Detailed Protocol). In comparison to the models results, empirical yields using the band intensities from (B) were plotted based on transcript length. (H) Effects of biotin concentration on modeled s^4U -RNA enrichment. Synthetic short RNAs (1 nM) with one s^4U residue (red) or zero s^4U residues (green) were enriched by 200 μM (comparable to 50 μg biotin in total RNA pulldown) or 20 μM HPDP- or MTS-biotin. No significant difference in enrichment was observed using these two concentrations of MTS-biotin eluent, whereas 200 μM HPDP-biotin showed 6-fold greater enrichment over 20 μM HPDP-biotin.

Figure S2. Reproducibility of MTS-biotin enrichment, related to Figure 2

(A) Whole genome alignments of RNA-Seq samples as in Figure 2B. The y -axis indicates the number of reads normalized to total number of *S. pombe* aligned reads. To compare coverage between samples using the same scale on the y -axis, in many cases read coverage exceeds the y -axis upper limit in Input (135 cases), MTS-biotin (127 cases) and HPDP-biotin (4 cases). Chromosomes are indicated below the mapped reads. (B) Scatter plots and Pearson correlations of normalized FPKM values for *H. sapiens* transcript isoforms. Plots show Input 1 vs. Input 2 (left), MTS-biotin replicate 1 vs. HPDP-biotin (center), and MTS-biotin replicate 1 vs. MTS-biotin replicate 2 (right). (C) Example of genes enriched by HPDP-biotin and MTS-biotin as in

Figure 2C. (D) Total reads for each RNA-Seq sample that mapped to the *H. sapiens* genome, normalized by total number of reads that mapped to the *S. pombe* genome, as in Figure 2D. (E) Samples enriched by MTS- or HPDP-biotin from RNA-seq submission were analyzed by qPCR using gene-specific primers for RPL18A, MOV10, HOXA9, CBX6, and UPF1 with two replicates. C_t values from qPCR were used to calculate percent input using the equation:

$$\frac{1}{2^{(C_{t_{sample}} - C_{t_{input}})}}$$

where the input is the average of two replicates. Error bars indicate the mean of two technical replicates +/- SEM. (F) C_t values from qPCR were plotted against the number of reads (\log_2 transformed) for deep sequencing in input (triangles), MTS- (circles) and HPDP- (squares) enriched samples.

Figure S3. Analysis of s^4U metabolic labeling and enrichment for miRNA RATE-Seq, related to Figure 3

(A-C) Scatter plots and Pearson correlations of RNA-Seq quantifications of *H. sapiens* miRNA transcripts. Plots show (A) reads from miRNA isolated from cells with not s^4U treatment compared to reads from total miRNA from cells after 22 days of s^4U treatment; (B) analysis of long RNAs from the same cells as in (A); and (C) analysis of miRNA isolated from 22 day s^4U treatment (10% input) vs. MTS-biotin enriched miRNA from 22 days of s^4U treatment. (D) miRNAs enriched with HPDP- and MTS-biotin. Control miRNA spikes containing one s^4U (EED004r) or zero s^4U (EED0095r) were enriched with s^4U -miRNA samples and enrichment was detected by qPCR using the same equation as above. The s^4U -containing spike-in control was not significantly enriched over background by HPDP-biotin ($p = 0.27$), whereas the s^4U -containing spike-in control was significantly enriched by MTS-biotin ($p = 0.034$). (E) Heatmap

similar to Figure 3C with annotated correlation coefficients (Pearson's r) between miRNA levels at different times after s^4U treatment. Replicate samples are indicated by (rep). (F) The enrichment of select miRNAs using MTS-biotin after 1 hr and 6 days s^4U treatment was validated by qPCR and quantified as fold enrichment as in Figure S2E. Error bars indicate the mean of three technical replicates +/- SEM.

Table S1. RNA-Seq alignment statistics

Table S2. Fast-turnover miRNAs that have previously been reported to be stable when transcription is inhibited. A list of miRNAs determined to be fast turnover (see Figure 3E), and their turnover rates (% turnover) in experiments where miRNA levels are monitored following transcriptional blockade (Bail et al., 2010; Guo et al., 2015).

Table S3. Oligonucleotide sequences. Names indicate internal spike codes (in the case of RNA synthetic spike), or target primer sequences (in the case of DNA qPCR primers). RNA synthetic spikes are synthetic RNA sequences containing either zero or one s^4U nucleotides that were included during s^4U -miRNA enrichment and sequencing (see Experimental Procedures). DNA qPCR primers were designed from sequences provided in Gregersen *et al.* 2014.

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