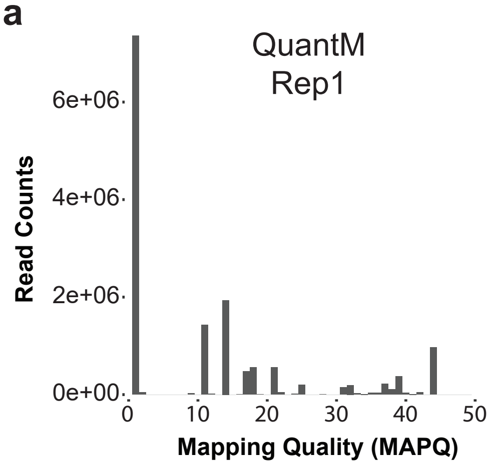
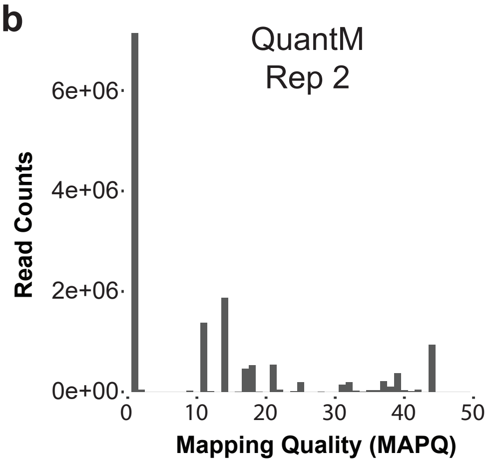
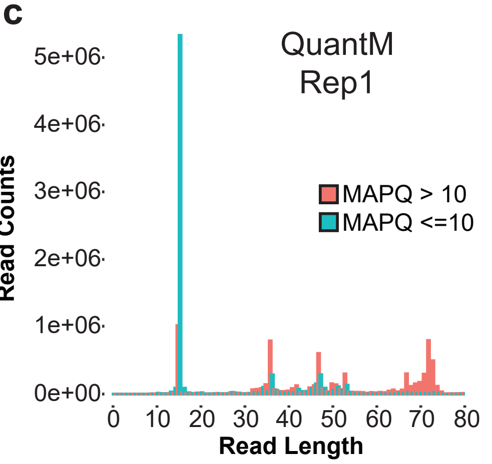
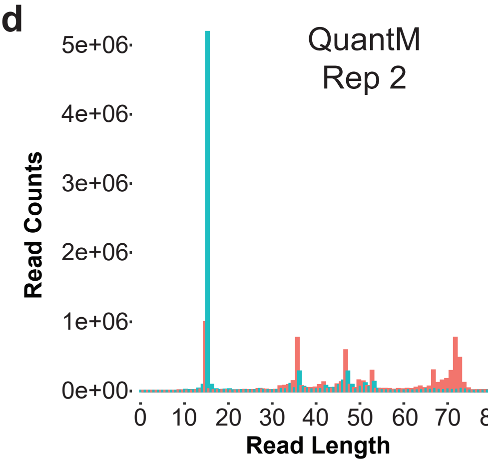
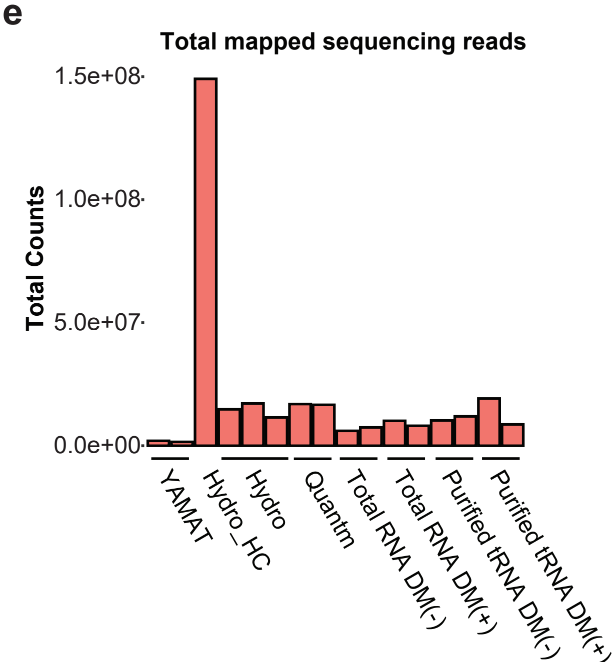
Pinkard et al Supplementary Figure 1

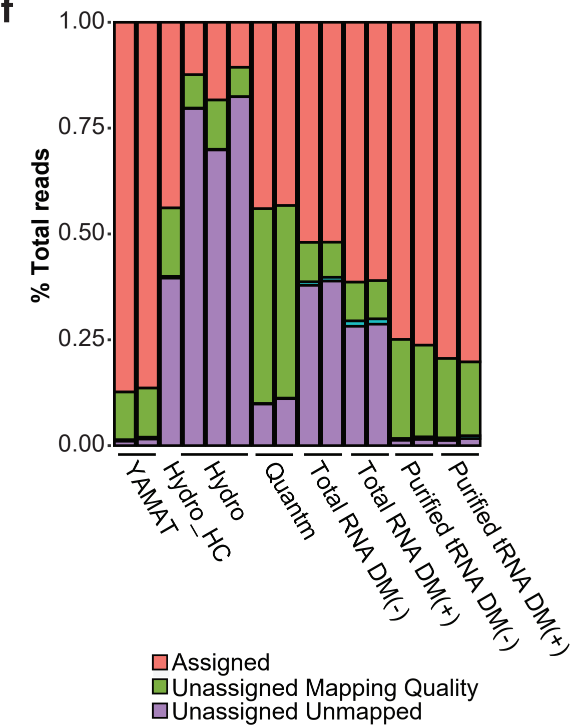




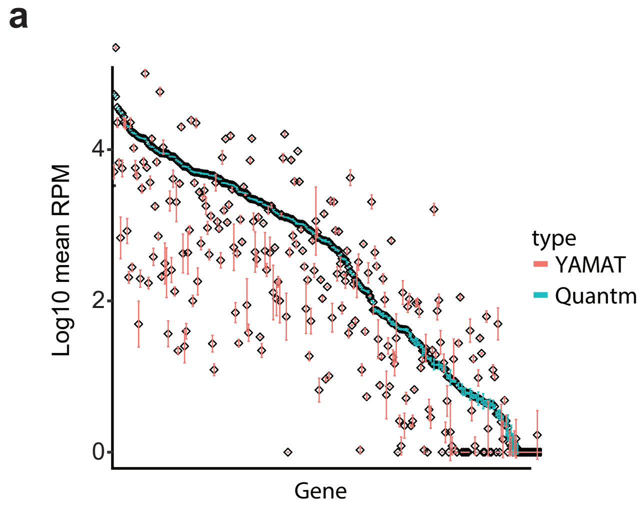


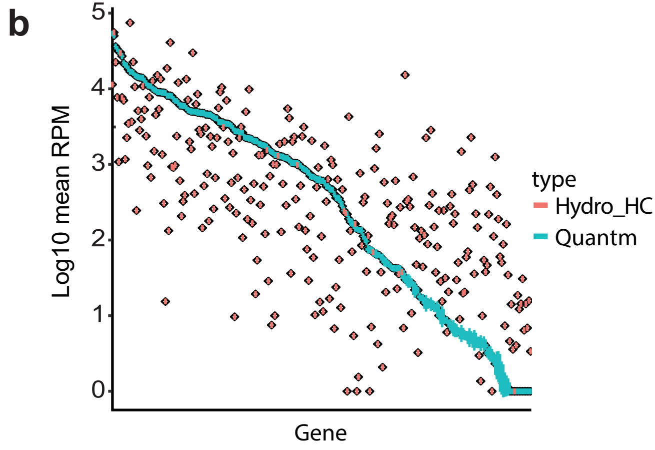


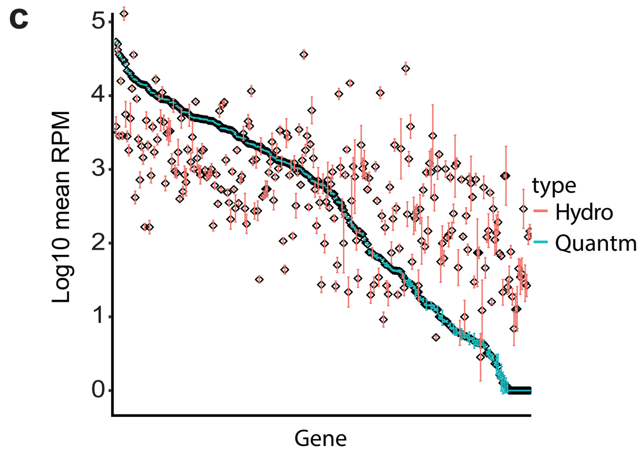


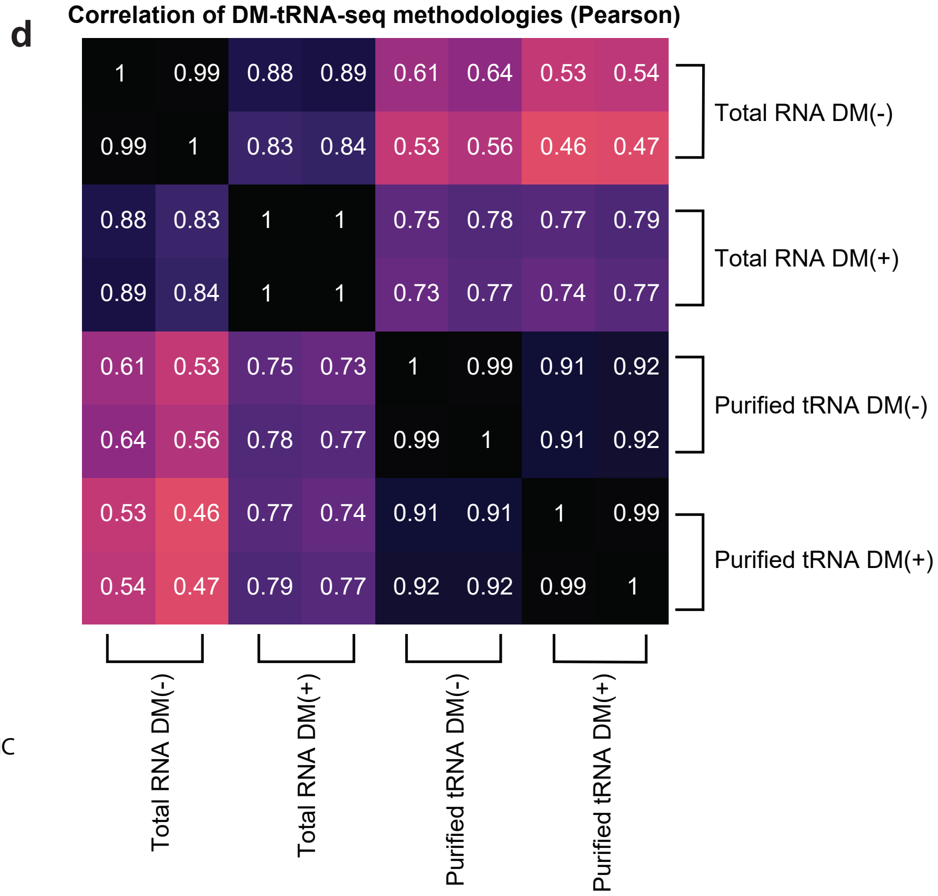


Pinkard et al Supplementary Data Figure 2

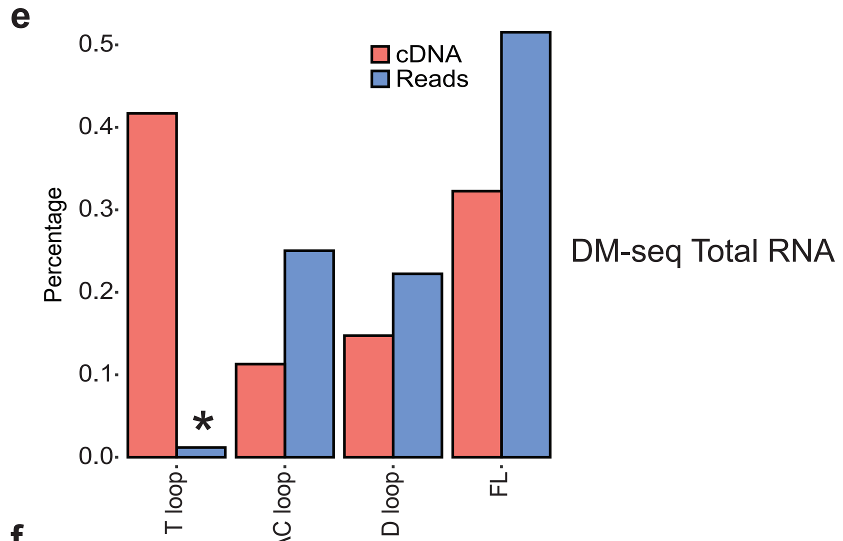


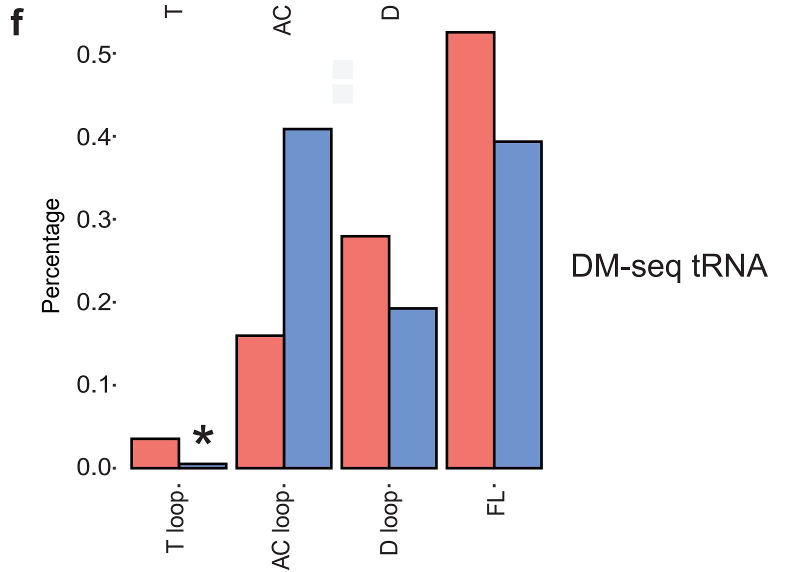




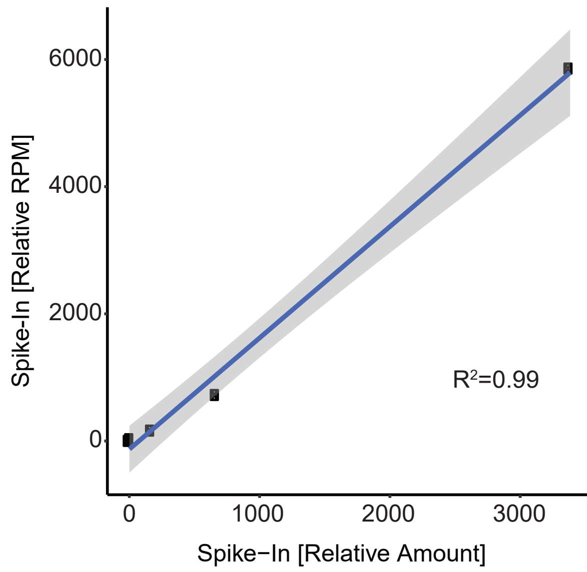


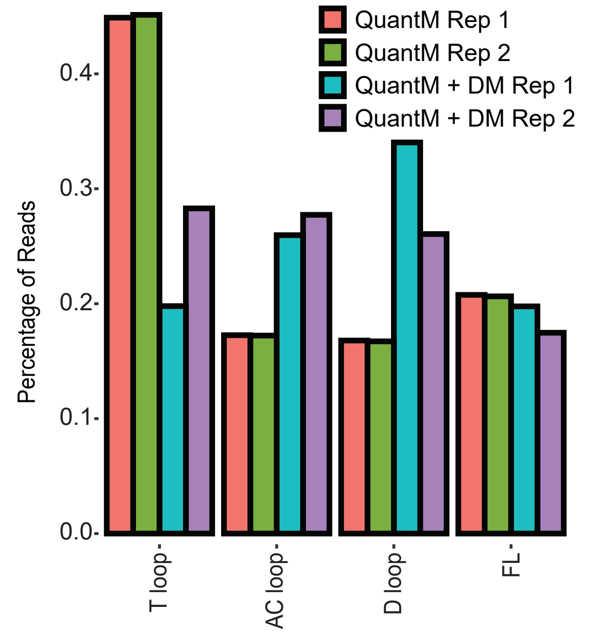
1	0.99	0.88	0.89	0.61	0.64	0.53	0.54
0.99	1	0.83	0.84	0.53	0.56	0.46	0.47
0.88	0.83	1	1	0.75	0.78	0.77	0.79
0.89	0.84	1	1	0.73	0.77	0.74	0.77
0.61	0.53	0.75	0.73	1	0.99	0.91	0.92
0.64	0.56	0.78	0.77	0.99	1	0.91	0.92
0.53	0.46	0.77	0.74	0.91	0.91	1	0.99
0.54	0.47	0.79	0.77	0.92	0.92	0.99	1

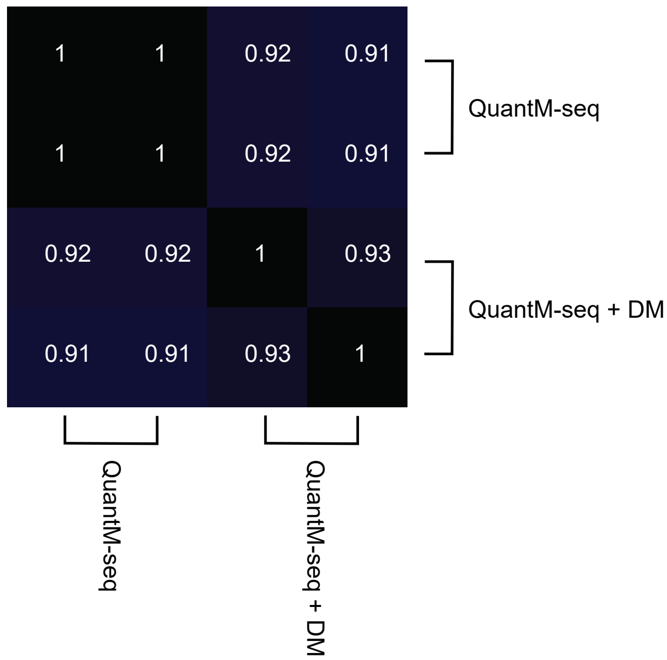


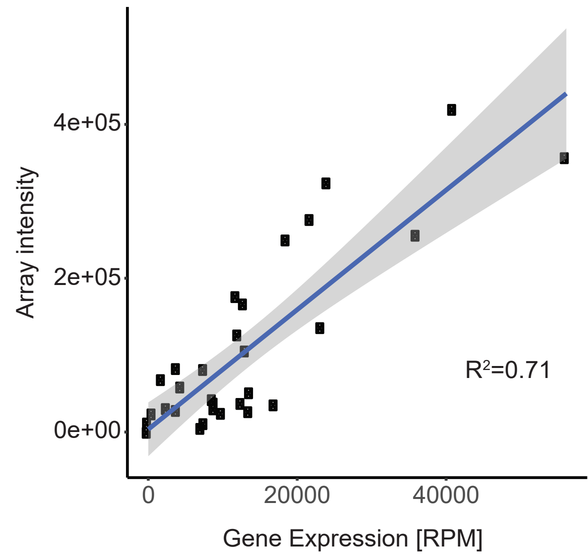


Pinkard et al Supplementary Figure 3



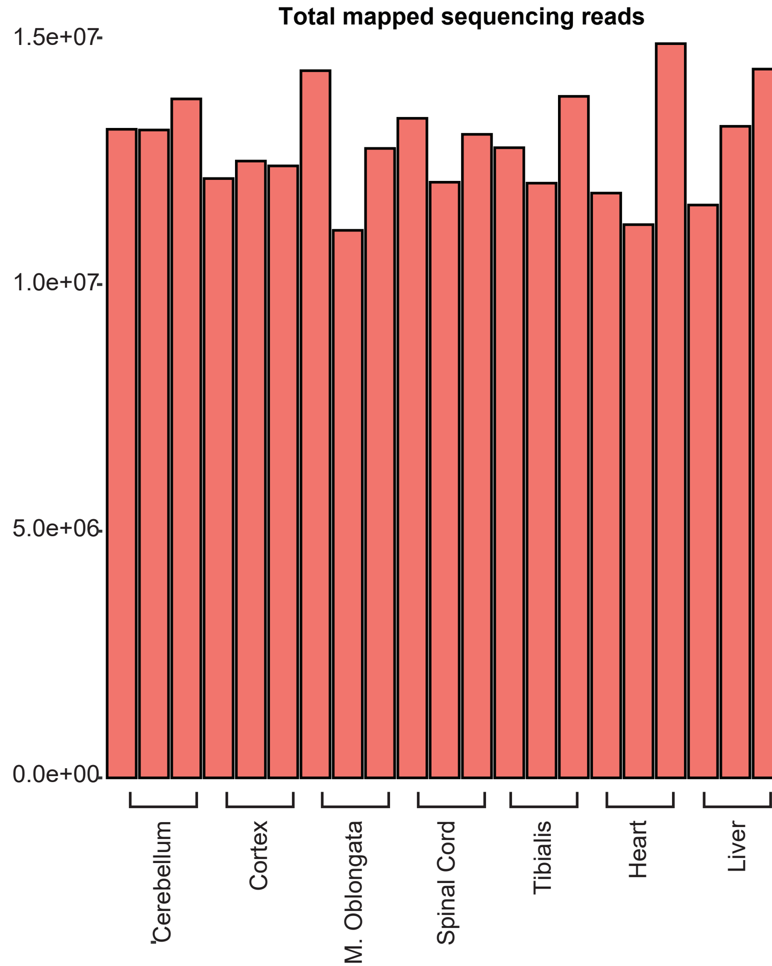






Pinkard et al Supplementary Figure 4

a



b

	Cerebellum			Cortex			M. Oblongata			Spinal Cord			Tibialis			Heart			Liver		
Cerebellum	1	1	0.96	0.96	0.99	0.97	0.99	0.99	0.94	0.99	0.99	0.99	0.97	0.98	0.97	0.97	0.98	0.99	0.97	0.97	0.97
	1	1	0.95	0.96	0.98	0.96	0.98	0.98	0.93	0.99	0.99	0.99	0.95	0.97	0.97	0.97	0.98	0.98	0.97	0.97	0.97
	0.96	0.95	1	0.91	0.95	0.99	0.96	0.97	0.98	0.96	0.96	0.97	0.92	0.96	0.97	0.96	0.94	0.96	0.93	0.94	0.94
Cortex	0.96	0.96	0.91	1	0.95	0.92	0.95	0.94	0.88	0.95	0.96	0.95	0.91	0.92	0.91	0.91	0.92	0.93	0.92	0.92	0.93
	0.99	0.98	0.95	0.95	1	0.97	0.99	0.99	0.92	0.99	0.99	0.99	0.97	0.97	0.97	0.97	0.97	0.98	0.97	0.97	0.97
	0.97	0.96	0.99	0.92	0.97	1	0.97	0.98	0.98	0.97	0.98	0.98	0.93	0.97	0.98	0.97	0.95	0.97	0.95	0.96	0.96
M. Oblongata	0.99	0.98	0.96	0.95	0.99	0.97	1	0.99	0.95	0.99	0.99	0.99	0.97	0.97	0.97	0.96	0.95	0.98	0.96	0.97	0.97
	0.99	0.98	0.97	0.94	0.99	0.98	0.99	1	0.95	0.99	0.99	0.99	0.97	0.98	0.97	0.97	0.96	0.98	0.97	0.97	0.97
	0.94	0.93	0.98	0.88	0.92	0.98	0.95	0.95	1	0.94	0.95	0.96	0.89	0.93	0.95	0.94	0.91	0.94	0.92	0.93	0.94
Spinal Cord	0.99	0.99	0.96	0.95	0.99	0.97	0.99	0.99	0.94	1	1	0.99	0.97	0.98	0.97	0.97	0.97	0.99	0.97	0.97	0.97
	0.99	0.99	0.96	0.96	0.99	0.98	0.99	0.99	0.95	1	1	1	0.97	0.98	0.98	0.98	0.98	0.98	0.98	0.98	0.98
	0.99	0.99	0.97	0.95	0.99	0.98	0.99	0.99	0.96	0.99	1	1	0.95	0.98	0.98	0.98	0.97	0.98	0.97	0.97	0.98
Tibialis	0.97	0.95	0.92	0.91	0.97	0.93	0.97	0.97	0.89	0.97	0.97	0.95	1	0.98	0.97	0.95	0.97	0.98	0.97	0.97	0.95
	0.98	0.97	0.96	0.92	0.97	0.97	0.97	0.98	0.93	0.98	0.98	0.98	0.98	1	0.99	0.98	0.98	0.99	0.97	0.97	0.97
	0.97	0.97	0.97	0.91	0.97	0.98	0.97	0.97	0.95	0.97	0.98	0.98	0.97	0.99	1	0.99	0.98	0.99	0.97	0.96	0.97
Heart	0.97	0.97	0.96	0.91	0.97	0.97	0.96	0.97	0.94	0.97	0.98	0.98	0.95	0.98	0.99	1	0.99	0.99	0.97	0.96	0.97
	0.98	0.98	0.94	0.92	0.97	0.95	0.95	0.96	0.91	0.97	0.98	0.97	0.97	0.98	0.98	0.99	1	0.99	0.98	0.97	0.98
	0.99	0.98	0.96	0.93	0.98	0.97	0.98	0.98	0.94	0.99	0.98	0.98	0.98	0.99	0.99	0.99	0.99	1	0.97	0.97	0.98
Liver	0.97	0.97	0.93	0.92	0.97	0.95	0.96	0.97	0.92	0.97	0.98	0.97	0.97	0.97	0.97	0.97	0.98	0.97	1	1	0.99
	0.97	0.97	0.94	0.92	0.97	0.96	0.97	0.97	0.93	0.97	0.98	0.97	0.97	0.97	0.96	0.96	0.97	0.97	1	1	0.99
	0.97	0.97	0.94	0.93	0.97	0.96	0.97	0.97	0.94	0.97	0.98	0.98	0.95	0.97	0.97	0.97	0.98	0.98	0.99	0.99	1

0.4 0.6 0.8 1

Pearson r−value

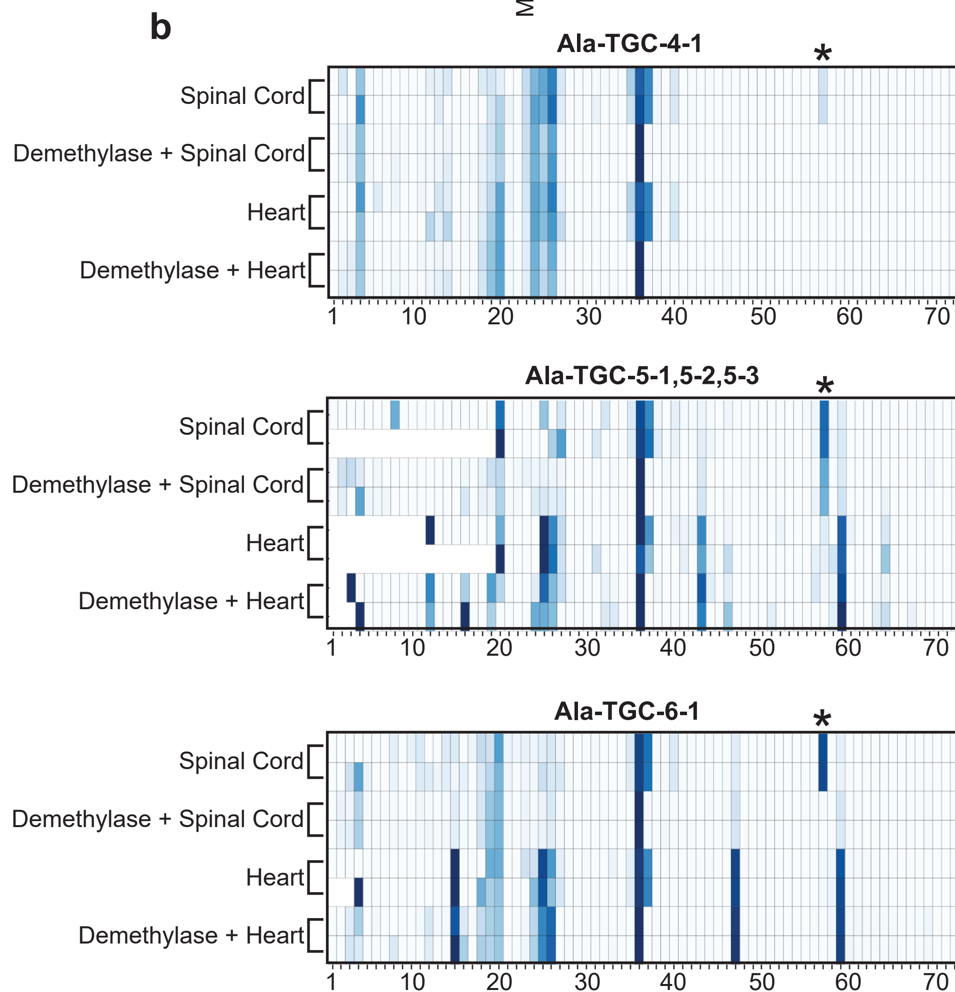
Pinkard et al Supplementary Figure 5

a

Pearson r−value: 0.4, 0.6, 0.8, 1

	Cerebellum			Cortex			M. Oblongata			Spinal Cord			Tibialis			Heart			Liver		
Cerebellum	1	0.97	0.96	0.89	0.97	0.96	0.97	0.96	0.96	0.97	0.96	0.96	0.94	0.95	0.95	0.95	0.94	0.94	0.94	0.95	0.95
	0.97	1	0.95	0.88	0.96	0.94	0.95	0.93	0.94	0.96	0.95	0.94	0.92	0.94	0.94	0.93	0.94	0.93	0.93	0.94	0.95
	0.96	0.95	1	0.89	0.97	0.99	0.97	0.97	0.98	0.97	0.97	0.97	0.93	0.93	0.95	0.95	0.94	0.95	0.95	0.94	0.94
Cortex	0.89	0.88	0.89	1	0.9	0.9	0.9	0.9	0.89	0.9	0.91	0.9	0.86	0.86	0.88	0.88	0.87	0.88	0.89	0.88	0.88
	0.97	0.96	0.97	0.9	1	0.97	0.97	0.97	0.96	0.97	0.97	0.97	0.93	0.94	0.95	0.95	0.95	0.95	0.96	0.95	0.95
	0.96	0.94	0.99	0.9	0.97	1	0.97	0.97	0.98	0.97	0.97	0.97	0.93	0.93	0.94	0.95	0.94	0.94	0.95	0.94	0.94
M. Oblongata	0.97	0.95	0.97	0.9	0.97	0.97	1	0.98	0.97	0.97	0.98	0.97	0.94	0.94	0.95	0.95	0.94	0.95	0.95	0.95	0.94
	0.96	0.93	0.97	0.9	0.97	0.97	0.98	1	0.97	0.97	0.98	0.98	0.93	0.93	0.95	0.95	0.93	0.94	0.95	0.94	0.93
	0.96	0.94	0.98	0.89	0.96	0.98	0.97	0.97	1	0.96	0.97	0.97	0.93	0.93	0.95	0.94	0.93	0.94	0.94	0.94	0.93
Spinal Cord	0.97	0.96	0.97	0.9	0.97	0.97	0.97	0.97	0.96	1	0.97	0.97	0.93	0.94	0.95	0.95	0.94	0.95	0.95	0.94	0.94
	0.96	0.95	0.97	0.91	0.97	0.97	0.98	0.98	0.97	0.97	1	0.98	0.94	0.94	0.95	0.95	0.94	0.94	0.96	0.94	0.94
	0.96	0.94	0.97	0.9	0.97	0.97	0.97	0.98	0.97	0.97	0.98	1	0.93	0.93	0.95	0.95	0.94	0.94	0.95	0.94	0.94
Tibialis	0.94	0.92	0.93	0.86	0.93	0.93	0.94	0.93	0.93	0.93	0.94	0.93	1	0.95	0.96	0.96	0.94	0.95	0.96	0.95	0.95
	0.95	0.94	0.93	0.86	0.94	0.93	0.94	0.93	0.93	0.94	0.94	0.93	0.95	1	0.97	0.96	0.96	0.97	0.96	0.96	0.96
	0.95	0.94	0.95	0.88	0.95	0.94	0.95	0.95	0.95	0.95	0.95	0.95	0.96	0.97	1	0.97	0.96	0.97	0.97	0.96	0.96
Heart	0.95	0.93	0.95	0.88	0.95	0.95	0.95	0.95	0.94	0.95	0.95	0.95	0.96	0.96	0.97	1	0.97	0.98	0.98	0.98	0.97
	0.94	0.94	0.94	0.87	0.95	0.94	0.94	0.93	0.93	0.94	0.94	0.94	0.94	0.96	0.96	0.97	1	0.97	0.97	0.96	0.96
	0.94	0.93	0.95	0.88	0.95	0.94	0.95	0.94	0.94	0.95	0.94	0.94	0.95	0.97	0.97	0.98	0.97	1	0.97	0.97	0.96
Liver	0.94	0.93	0.95	0.89	0.96	0.95	0.95	0.95	0.94	0.95	0.96	0.95	0.96	0.96	0.97	0.98	0.97	0.97	1	0.97	0.96
	0.95	0.94	0.94	0.88	0.95	0.94	0.95	0.94	0.94	0.94	0.94	0.94	0.95	0.96	0.96	0.98	0.96	0.97	0.97	1	0.96
	0.95	0.95	0.94	0.88	0.95	0.94	0.94	0.93	0.93	0.94	0.94	0.94	0.95	0.96	0.96	0.97	0.96	0.96	0.96	0.96	1

b



Supplementary Figure Legends

Supplementary Figure 1: Sequencing metrics of QuantM-seq from HEK293 cells. Read count histograms of mapping quality scores (MAPQ) derived from QuantM-seq of HEK 293 cells for biological replicate 1 (**a**) and biological replicate 2 (**b**). Bar charts of read lengths vs. read counts classified as MAPQ > 10 or MAPQ <= 10 for QuantM-seq of HEK 293 cells for biological replicate 1 (**c**) and biological replicate 2 (**d**). **e,** A histogram depicting the total mapped reads (MAPQ > 10) for YAMAT-seq (YAMAT), Hydro-tRNA-seq with high coverage (Hydro_HC), Hydro-tRNA-seq with lower coverage (Hydro), QuantM-tRNA-seq (QuantM), DM-tRNA-seq performed on PAGE purified tRNA +/- pretreatment with demethylase (Total RNA DM +/-), DM-tRNA-seq performed on total RNA +/- pretreatment with demethylase (Purified tRNA DM +/-). Each bar represents a separate biological replicate for each methodology performed on HEK293 cells. **f,** Fraction of reads Assigned (MAPQ > 10), Unassigned Mapping Quality (MAPQ <= 10), or unmapped. Each bar represents a separate biological replicate for each methodology performed on HEK293 cells.

Supplementary Figure 2: Comparison of QuantM-seq with other tRNA-seq protocols, including a high output Hydro-seq library. Plots of $\log_{10}$-transformed reads per million for unique tRNA sequences. QuantM-seq is always represented by cyan dots compared to YAMAT-seq (N=2 biological replicates) (**a**), Hydro_HC high coverage hydro-seq library (N=1 sample) (**b**), or lower coverage Hydro-seq libraries (N=3 biological replicates) (**c**) in red. Error bars represent standard deviation of

replicates. **d,** Pearson correlation coefficients between gene-level expression for each dataset. Total RNA DM(-): DM-tRNA-seq performed on total RNA without demethylase treatment, Total RNA DM(+): DM-tRNA-seq performed on total RNA with demethylase treatment, Purified tRNA DM(-): DM-tRNA-seq performed on gel purified tRNA without demethylase treatment, Purified tRNA DM(+): DM-tRNA-seq performed on gel purified tRNA with demethylase treatment. Bar charts of percentage of reads from DM-tRNA-seq on total RNA (**e**) or purified tRNA (**f**) where reverse transcriptase stalls or falls of in the T-loop, anticodon (AC) loop, D-loop, or at the end of tRNA (full length; FL). Red and Blue bars represent two separate biological replicates. cDNA represents values derived from densitometry of cDNA gels, reads are values from sequencing reads. * - denotes short reads (15 nt) that represent stalling at m1A in the T-loop clearly evident in cDNA were discarded from the GEO record.

Supplementary Figure 3: Demethylase treatment prior to QuantM-seq does not detectably improve quantitation. a, Scatter plot of average relative amount of *in vitro* transcribed *E. coli* tRNAs spiked into two HEK293 QuantM-seq libraries versus amounts detected by sequencing in reads per million. Shaded area represents the 95% confidence interval of the linear trendline. **b,** Bar chart of percentage reads from QuantM-seq of RNA from HEK293 cells treated with or without demethylase (DM) where reverse transcriptase stalls or falls off in the T-loop, anticodon (AC) loop, D-loop, or at the end of tRNA (full length; FL). Each bar represents individual biological replicates. **c,** Pearson correlation coefficients between QuantM-seq libraries with and without demethylase treatment (DM - demethylase). **d,** Scatter plot of expression values

from QuantM-seq performed on HEK293 RNA treated with demethylase versus tRNA array intensities. Shaded area represents the 95% confidence interval of the linear trendline.

Supplementary Figure 4: QuantM-seq of RNA from mouse tissues. a, Bar graph depicting sequencing depth for each mouse tissue tRNA dataset analyzed. **b,** Pearson correlation coefficients for isodecoder-level expression between each mouse tissue.

Supplementary Figure 5: Variants in sequencing change across tissues in tRNA bases known to be modified. a, Pearson correlation coefficients between isodecoder-level variant fractions for each mouse tissue. Only tRNA bases with variant fractions that were >1% in all tissue samples were considered as potential sequence variants. **b,** Heatmaps for three isodecoders representing variant fractions at each tRNA position (x-axis) across tissue samples (y-axis). RNA from a CNS tissue (spinal cord) and a non-CNS tissue (heart) were either treated or untreated with demethylase before QuantM-seq. Note that position 56 (*), which varies only in CNS tissues (Fig. 6), loses variation upon treatment with demethylase, indicating that the m^1A was methyl group was removed. The numbers below the plot indicate nucleotide position.