

Supplemental Information

A new reagent for *in vivo* structure probing of RNA G and U residues
that improves RNA structure prediction alone and combined with DMS

Catherine A Douds^{1,2}, Paul Babitzke^{1,2}, Philip C. Bevilacqua^{1,2,3}

1. Department of Biochemistry and Molecular Biology, Pennsylvania State University,
University Park, Pennsylvania 16802, USA
2. Center for RNA Molecular Biology, Pennsylvania State University, University Park,
Pennsylvania 16802, USA
3. Department of Chemistry, Pennsylvania State University, University Park, Pennsylvania
16802, USA

Corresponding author: pcb5@psu.edu

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Supplemental Table S1: P-values for Supplemental Fig. S5.

	TGIRT-III 65°C		TGIRT-III 70°C		Marathon		SuperScript II		HIV RT	
A	1.35E-02	*	1.07E-14	****	2.60E-05	****	6.38E-02	ns	2.98E-43	****
C	5.02E-01	ns	2.59E-07	****	4.50E-02	*	9.41E-01	ns	7.65E-51	****
G	8.97E-17	****	5.84E-19	****	1.42E-12	****	9.13E-06	****	7.65E-150	****
U	1.04E-95	****	2.70E-87	****	2.40E-46	****	8.98E-46	****	3.15E-150	****

Wilcox test comparing distributions of raw mutation rates between untreated and treated samples for each nucleotide and RT condition in the pilot experiments for ETC reactions of *E. coli* 16S rRNA and 23S rRNA with MaP readout. The significance reported for each asterisk is in the Materials and Methods.

Supplemental Table S2: Medians for Fig. 3.

	TGIRT-III 65°C		TGIRT-III 70°C		Marathon		SuperScript II		HIV RT	
	Paired	Unpaired	Paired	Unpaired	Paired	Unpaired	Paired	Unpaired	Paired	unpaired
A	3.60E-05	1.90E-05	6.00E-05	7.40E-05	5.20E-05	6.80E-05	2.00E-06	1.30E-05	3.38E-04	3.38E-04
C	6.00E-06	1.50E-05	4.70E-05	7.00E-05	2.60E-05	5.00E-05	-1.80E-05	-1.10E-05	4.59E-04	5.09E-04
G	1.06E-04	2.97E-04	2.57E-04	3.91E-04	1.11E-04	2.85E-04	9.10E-05	2.26E-04	1.14E-03	1.27E-03
U	1.53E-04	6.13E-04	1.14E-04	3.78E-04	9.00E-05	3.06E-04	1.21E-04	5.43E-04	6.29E-04	8.23E-04

Medians for background subtracted mutation rates distributions of pilot experiments of ETC reactions of *E. coli* 16S rRNA and 23S rRNA with MaP readout.

Supplemental Table S3: P-values for Fig. 3.

	TGIRT-III 65°C		TGIRT-III 70°C		Marathon		SuperScript II		HIV RT	
A	1.00E+00	ns	8.00E-02	ns	8.40E-02	ns	9.33E-01	ns	1.00E+00	ns
C	4.46E-01	ns	1.68E-01	ns	1.10E-01	ns	4.46E-01	ns	1.68E-01	ns
G	2.91E-33	****	1.95E-17	****	1.46E-18	****	2.24E-14	****	5.00E-03	**
U	1.88E-50	****	4.04E-37	****	5.96E-29	****	8.64E-36	****	7.69E-08	****

Wilcox test comparing distributions of background-subtracted mutation rates between paired and unpaired nucleotides for each nucleotide and RT condition in the pilot experiments for ETC reactions of *E. coli* 16S rRNA and 23S rRNA with MaP readout. The significance reported for each asterisk is in the Materials and Methods.

Supplemental Table S4: Medians and p-values for Fig. 4A.

	DMS				ETC			
	Paired median	Unpaired median	p-value	significance	Paired median	Unpaired median	p-value	significance
A	3.26E-03	7.17E-03	4.68E-18	****	3.6E-05	1.90E-05	1.00E+00	ns
C	3.70E-04	4.19E-03	5.97E-65	****	6.00E-06	1.50E-05	3.08E-01	ns
G	7.40E-04	6.76E-04	3.00E-03	**	1.06E-04	2.97E-04	1.75E-33	****
U	1.14E-04	2.05E-04	3.47E-15	****	1.53E-04	6.13E-04	7.50E-51	****

Medians for background-subtracted mutation rate distributions as well as p-values for Wilcoxon test comparing distributions of paired and unpaired rates for each nucleotide with DMS and ETC in MaP libraries of *E. coli* 16S rRNA and 23S rRNA made using TGIRT-III with extension at 65°C. The significance reported for each asterisk is in the Materials and Methods. Values in this table were calculated on the full data, while the logarithmic plots in Fig. 4A are missing zeros and so show slightly different medians in some cases.

Supplemental Table S5: Medians for Fig. 4B.

	UT	1X ETC	1.5X ETC
A	6.00E-04	6.40E-04	6.56E-04
C	6.13E-04	6.29E-04	6.11E-04
G	1.21E-03	1.41E-03	1.31E-03
U	4.17E-04	7.59E-04	6.55E-04

Medians of mutation rates for untreated (UT), 200 mM ETC (1X) and 300 mM ETC (1.5X) pilot MaP libraries of *E. coli* 16S rRNA and 23S rRNA made using TGIRT-III with extension at 65°C. Values in this table were calculated on the full data, while the logarithmic plots in Fig. 4B are missing zeros and so show slightly different medians in some cases.

Supplemental Table S6: P-values for Fig. 4B.

	UT vs 1X ETC		UT vs 1.5X ETC		1X ETC vs 1.5X ETC	
A	3.50E-02	*	2.40E-02	*	1.00E+00	ns
C	1.00E+00	ns	1.00E+00	ns	1.00E+00	ns
G	3.59E-16	****	1.54E-05	****	1.31E-03	**
U	4.16E-95	****	2.73E-66	****	5.53E-04	***

Wilcoxon test comparing distributions of raw mutation rates between untreated (UT), 200 mM ETC (1X) and 300 mM ETC (1.5X) pilot MaP libraries of *E. coli* 16S rRNA and 23S rRNA made using TGIRT-III with extension at 65°C. The significance reported for each asterisk is in the Materials and Methods. Values in this table were calculated on the full data.

Supplemental Table S7: P-values for Supplemental Fig. S7A.

	UT 50°C v 55°C		UT 50°C v 60°C		UT 55°C 60°C	
A	1.47E-12	****	4.00E-03	**	3.49E-23	****
C	8.55E-07	****	1.40E-02	*	1.36E-13	****
G	1.25E-08	****	4.25E-12	****	2.20E-36	****
U	3.13E-05	****	4.32E-04	***	2.29E-15	****

Wilcox test comparing distributions of raw mutation rates between untreated libraries with RT extension at 50°C, 55°C, and 60°C. The significance reported for each asterisk is in the Materials and Methods. Values in this table were calculated on the full data, while the logarithmic plots in Supplemental Fig. S7A are missing zeros and so show slightly different medians in some cases.

Supplemental Table S8: P-values for Supplemental Fig. S7B.

	50°C		55°C		60°C	
A	3.30E-16	****	5.98E-05	****	5.49E-14	****
C	2.87E-09	****	4.42E-09	****	6.50E-06	****
G	2.68E-12	****	1.03E-24	****	2.00E-02	*
U	2.34E-52	****	1.67E-17	****	6.25E-25	****

Wilcox test comparing distributions of raw mutation rates between untreated and ETC-treated libraries with RT extension at 50°C, 55°C, and 60°C. The significance reported for each asterisk is in the Materials and Methods. Values in this table were calculated on the full data, while the logarithmic plots in Supplemental Fig. S7B are missing zeros and so show slightly different medians in some cases.

Supplemental Table S9: Medians for Fig. 5A,B.

	50°C		55°C		60°C	
	Paired	Unpaired	Paired	Unpaired	Paired	unpaired
A	9.25E-05	1.04E-04	-4.10E-05	-4.80E-05	9.37E-05	1.04E-04
C	6.20E-05	9.80E-05	-5.65E-05	-5.5E-05	4.84E-05	8.71E-05
G	2.99E-04	4.38E-04	4.53E-04	6.29E-04	-1.54E-04	-7.75E-05
U	9.20E-05	3.16E-04	1.00E-05	2.00E-04	6.33E-05	2.95E-04

Medians for background-subtracted mutation rates distributions of paired and unpaired residues for libraries with RT extension at 50°C, 55°C, and 60°C.

Supplemental Table S10: P-values for Fig. 5A,B.

	50°C		55°C		60°C	
A	1.48E-01	ns	9.65E-01	ns	4.86E-01	ns
C	2.86E-04	***	5.00E-01	ns	2.88E-05	****
G	9.51E-18	****	2.74E-10	****	6.41E-09	****
U	4.74E-47	****	1.95E-44	****	5.86E-45	****

Wilcox test comparing distributions of background-subtracted mutation rates between paired and unpaired residues for libraries with RT extension at 50°C, 55°C, and 60°C. The significance reported for each asterisk is in the Materials and Methods.

Supplemental Table S11: Average AUC values for biological replicates comparing across temperatures for Fig. 5C,D.

	Replicate A	Replicate B	Replicate C	Average AUC	Standard Deviation	Range
50°C U	0.78	0.77	0.75	0.76	0.016	0.747 - 0.779
55°C U	0.75	0.74	0.73	0.74	0.011	0.728 - 0.750
60°C U	0.75	0.76	0.73	0.75	0.013	0.735 - 0.763
50°C G	0.66	0.66	0.60	0.64	0.031	0.610 - 0.672
55°C G	0.64	0.60	0.57	0.60	0.037	0.565 - 0.640
60°C G	0.57	0.62	0.56	0.59	0.033	0.553 - 0.620

Area Under the Curve (AUC) values for receiver operating curves (ROC) of each biological replicate's background-subtracted mutation rates as a predictor of pairing state for libraries with TGIRT-III extension at 50°C, 55°C, and 60°C. The difference in AUC is not significant when comparing extension temperatures.

Supplemental Table S12: Medians for Fig. 6A and Supplemental Fig. S8A.

	T>A	T>G	T>C	1nt deletion	multinuc mismatch	multinuc deletion	multinuc insertion	complex deletion	complex insertion
paired	2.65E-04	2.47E-04	5.35E-04	2.36E-05	2.69E-04	1.33E-05	1.65E-06	4.10E-05	2.14E-05
unpaired	3.02E-04	3.02E-04	6.77E-04	3.52E-05	3.33E-04	2.50E-05	2.11E-06	5.17E-05	2.48E-05

Medians of background-subtracted mutation rate distributions for paired and unpaired Us at various mutation types with libraries made using TGIRT-III with extension at 50 °C. Values in this table were calculated on the full data, while the logarithmic plots in Fig. 6 and Supplemental Fig. S8A are missing zeros and so show slightly different medians in some cases.

Supplemental Table S13: P-values for Fig. 6A and Supplemental Fig. S8A.

T>A	T>G	T>C	1nt deletion	multinuc mismatch	multinuc deletion	multinuc insertion	complex deletion	complex insertion
1.45E-08 ****	3.30E-07 ****	4.57E-24 ****	3.16E-11 ****	2.46E-06 ****	2.88E-25 ****	8.14E-03 **	3.96E-17 ****	9.15E-09 ****

Wilcox test comparing distributions of background-subtracted mutation rates between paired and unpaired Us at various mutation types with libraries made using TGIRT-III with extension at 50 °C. The significance reported for each asterisk is in the Materials and Methods. Values in this table were calculated on the full data.

Supplemental Table S14: Medians for Fig. 6A and Supplemental Fig. S8B.

	G>C	G>T	G>A	1nt deletion	multinuc mismatch	multinuc deletion	multinuc insertion	complex deletion	complex insertion
paired	1.43E-04	2.26E-04	2.20E-03	8.54E-06	2.88E-04	1.26E-05	7.76E-06	4.74E-05	3.65E-05
unpaired	1.79E-04	2.80E-04	2.41E-03	2.00E-05	2.88E-04	1.41E-05	6.85E-06	5.06E-05	4.15E-05

Medians of background-subtracted mutation rate distributions for paired and unpaired Gs at various mutation types with libraries made using TGIRT-III with extension at 50 °C. Values in this table were calculated on the full data, while the logarithmic plots in Fig. 6 and Supplemental Fig. S8B are missing zeros and so show slightly different medians in some cases.

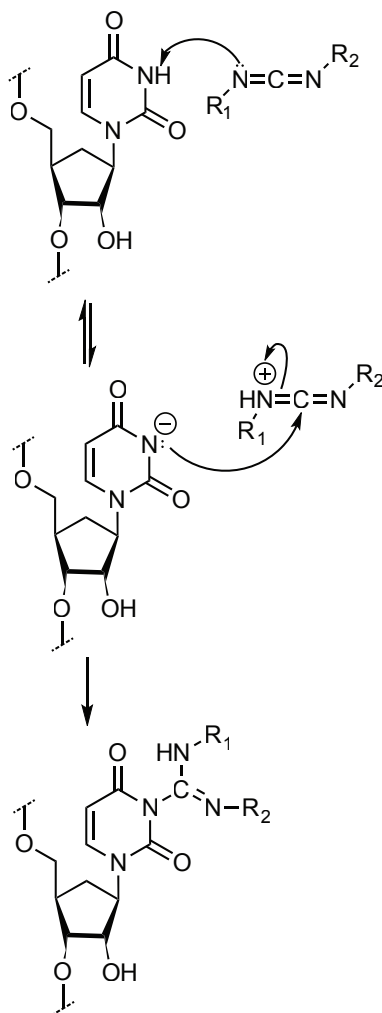
Supplemental Table S15: P-values for Fig. 6A and Supplemental Fig. S8B.

G>C	G>T	G>A	1nt deletion	multinuc mismatch	multinuc deletion	multinuc insertion	complex deletion	complex insertion
2.62E-05 ****	3.92E-09 ****	1.31E-02 *	1.62E-05 ****	8.64E-01 ns	1.31E-02 *	8.64E-01 ns	2.19E-01 ns	2.19E-01 ns

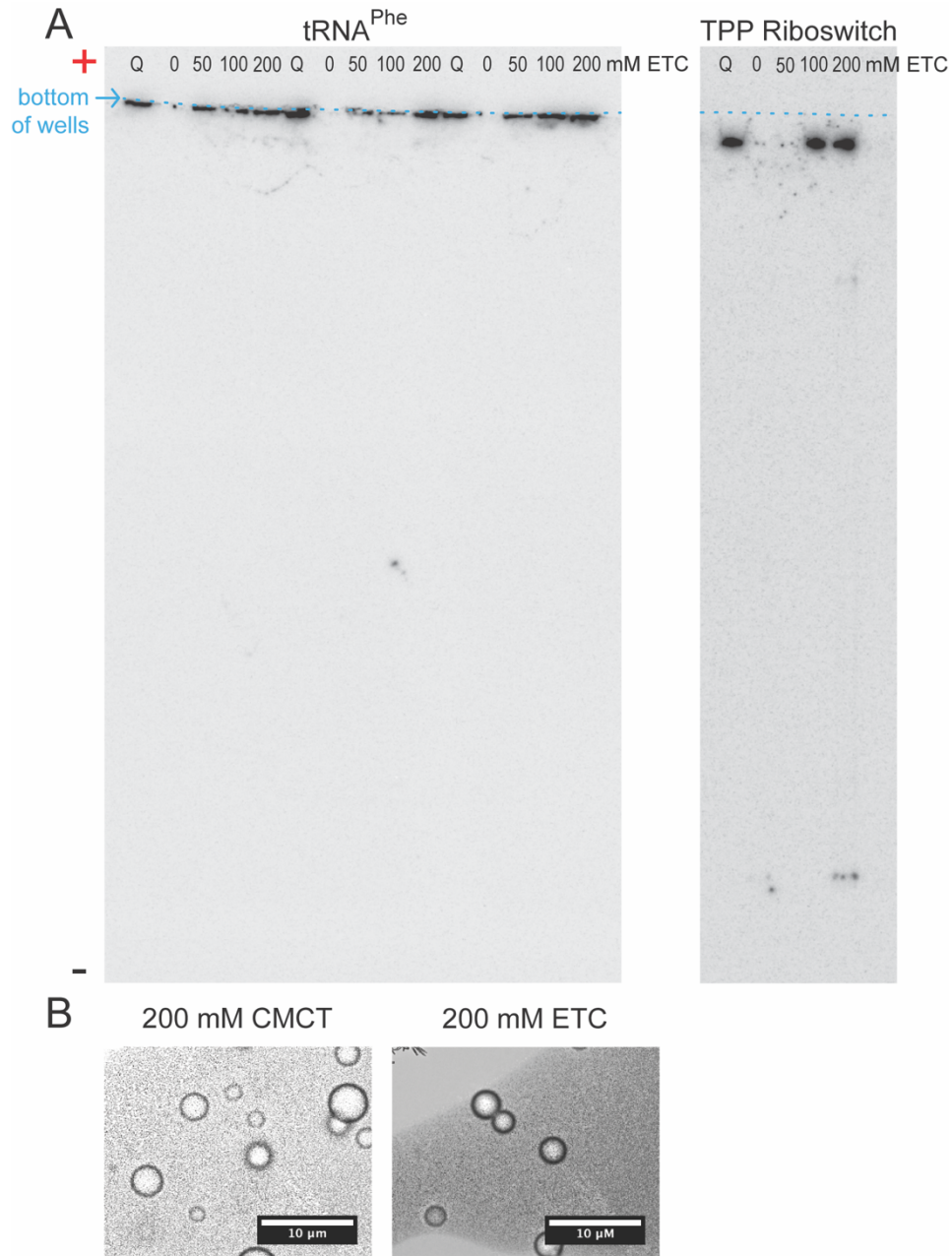
Wilcox test comparing distributions of background-subtracted mutation rates between paired and unpaired Gs at various mutation types with libraries made using TGIRT-III with extension at 50 °C. The significance reported for each asterisk is in the Materials and Methods. Values in this table were calculated on the full data.

Supplemental Table S16: Total reads and mapping statistics for sequencing libraries

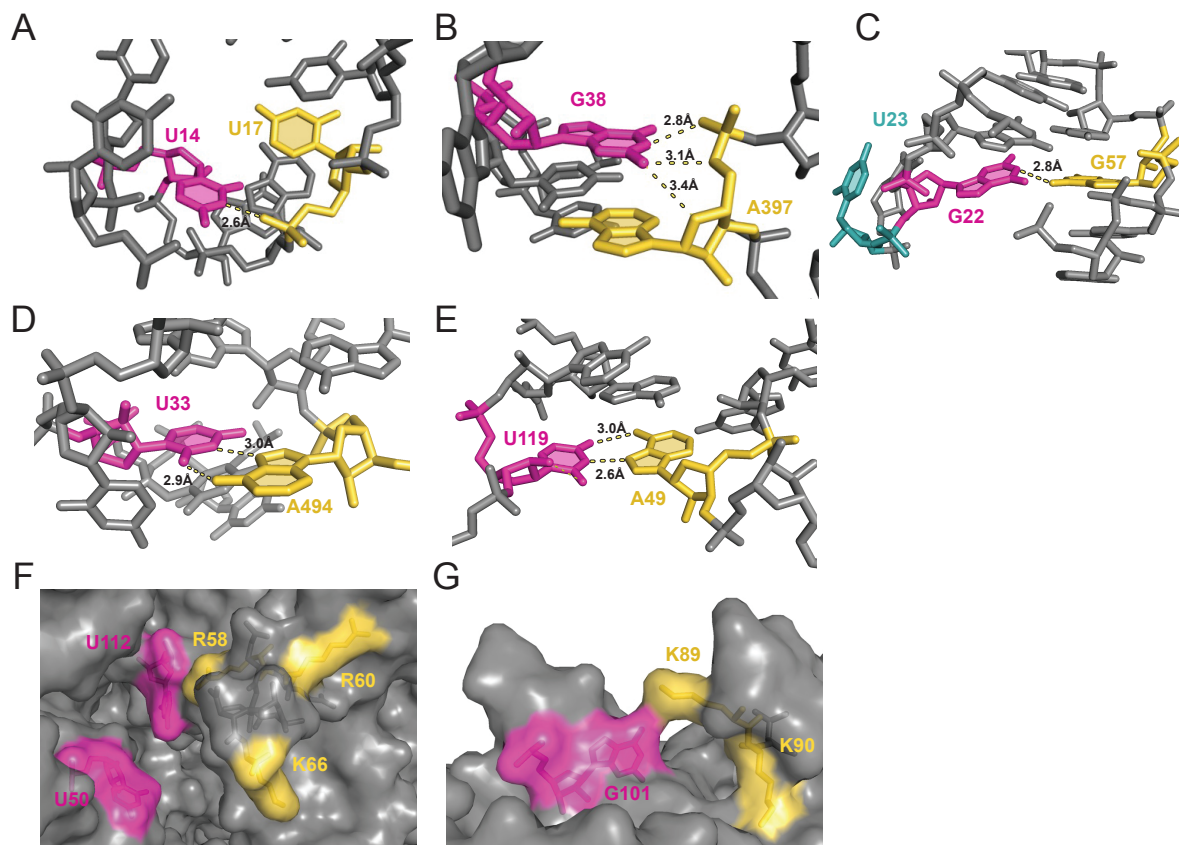
RT condition	treatment	replicate	total reads	average insert (nt)	overall alignment rate
TGIRT-III at 65°C	untreated	1	10,338,765	186.5	94.74%
	200 mM ETC	1	12,431,321	185.0	96.38%
	300 mM ETC	1	14,272,865	183.3	95.84%
	100 mM DMS	1	11,826,074	187.2	95.33%
TGIRT-III at 70°C	untreated	1	10,905,156	192.0	92.23%
	200 mM ETC	1	11,976,565	181.4	96.51%
Marathon RT	untreated	1	9,330,550	177.2	93.67%
	200 mM ETC	1	13,216,223	167.4	95.00%
SuperScript II	untreated	1	13,053,109	161.8	96.23%
	200 mM ETC	1	4,560,860	161.8	97.57%
HIV RT	untreated	1	11,686,115	168.7	96.27%
	200 mM ETC	1	5,424,628	157.7	96.16%
TGIRT-III at 50°C	untreated	1	11,757,002	214.4	93.63%
		2	17,447,414	213.3	93.97%
		3	15,433,504	203.0	93.71%
	200 mM ETC	1	6,972,045	205.0	94.53%
		2	8,272,226	206.5	94.91%
		3	15,961,610	208.9	94.61%
TGIRT-III at 55°C	untreated	1	10519916	195.8	93.09%
		2	9,704,085	214.8	93.52%
		3	10,687,418	200.8	93.19%
	200 mM ETC	1	8,639,718	208.8	94.43%
		2	23,163,188	216.6	96.43%
		3	9,169,385	213.0	96.39%
	100 mM DMS	1	12,246,657	201.9	94.27%
		2	11,584,514	203.4	93.35%
		3	8,659,698	206.5	94.13%
TGIRT-III at 60°C	untreated	1	14,387,559	220.1	93.74%
		2	10,062,641	218.7	93.82%
		3	8,315,085	209.7	93.33%
	200 mM ETC	1	5,855,170	217.0	94.92%
		2	6,220,657	218.4	95.06%
		3	8,043,651	221.2	95.16%



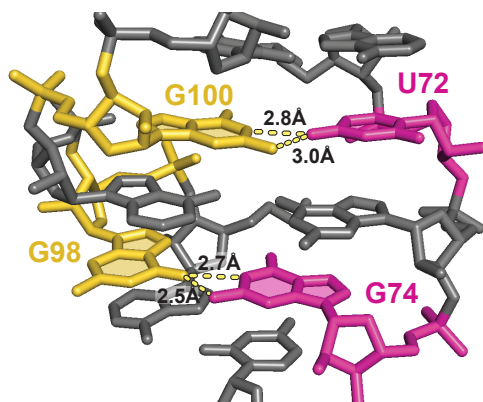
Supplemental Fig. S1: Reaction scheme for carbodiimide modification of the imino group. First, the carbodiimide abstracts a proton from the imino nitrogen. The resulting lone electron pair then acts as a nucleophile and attacks the central carbon of the carbodiimide, with the cationic amine acting as an electron sink. This reaction leads to a covalent attachment of the carbodiimide to the base. U is shown here, but G reacts similarly with EDC. We drew the most parsimonious mechanism for proton transfer between ETC and uracil. It remains possible that buffer or other small molecules could participate in proton transfer and that multiple reaction channels could populate.



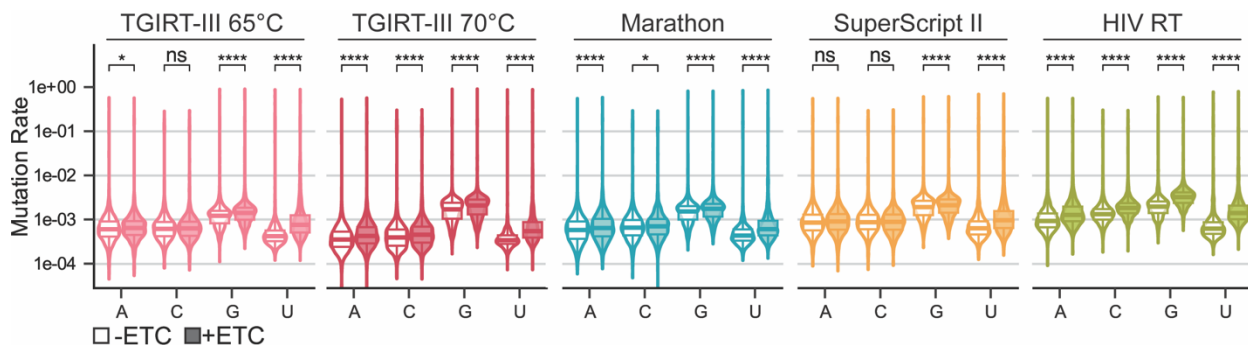
Supplemental Fig. S2: Evidence of phase separation for *in vitro* ETC and CMCT reactions. A) Reverse electrophoresis of *in vitro* treated ETC samples on a 10% denaturing PAGE where electrodes were reversed such that the negative electrode was at the bottom of the gel and the positive electrode was at the top. The bottom of the wells are marked with a dotted blue line. (Left) 5' end labeled $tRNA^{Phe}$ and (Right) 5' end labeled *E. coli* TPP riboswitch. We speculate that empty lanes on the gel for untreated reactions migrated into the top buffer, as evidenced by radioactivity in the top buffer. B) Microscope images of quenched CMCT and ETC *in vitro* reactions with $tRNA^{Phe}$. Both reactions showed the formation of droplets; however, only ETC droplets resulted in reverse electrophoresis. We speculate that owing to the differences in the carbodiimides, the interactions with RNA and thus droplet characteristics are different between chemicals.



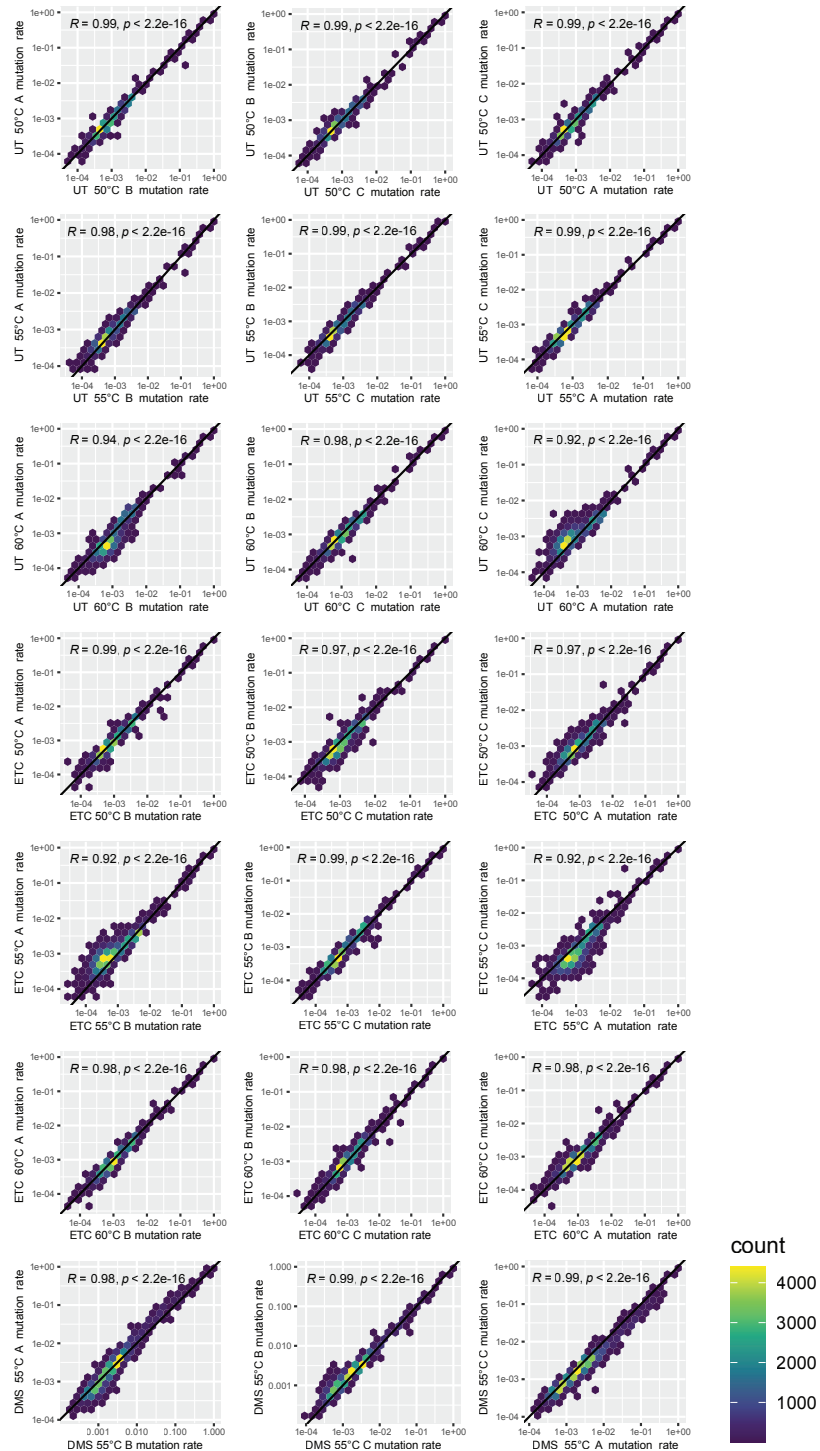
Supplemental Fig. S3: Structural basis for unreactive unpaired (false negative) nucleotides. False negative nucleotides are colored magenta, while interacting nucleotides are colored yellow. (A) *E. coli* 16S rRNA, unreactive N3 of U14 (magenta) forms a hydrogen bond with the nonbridging oxygen of U17 (yellow). (B) *E. coli* 16S rRNA, unreactive N1 of G38 (magenta) forms a hydrogen bond with the nonbridging oxygen and bridging oxygen of A397 (yellow). Additional stabilizing hydrogen bonds for the N2 of G38 to A397 are indicated. (C) *B. subtilis* 5S rRNA, unreactive N1 of G22 (magenta) forms a hydrogen bond with the O6 of G57 (yellow) and stacks with neighboring G21. In contrast, in the same internal loop, U23 (cyan) is fully accessible, consistent with its high reactivity. (D) *B. subtilis* 23S rRNA, unreactive U33 (magenta) forms two hydrogen bonds with the Hoogsteen face of A494 (yellow). (E) *B. subtilis* 23S rRNA, unreactive U119 (magenta) forms two hydrogen bonds with the Hoogsteen face of A49 (yellow). (F) *B. subtilis* 23S rRNA, unreactive U50 and U112 (magenta) are occluded by three positively charged amino acids R58, R60, and K66 (yellow) in ribosomal protein L29. (G) *B. subtilis* 23S rRNA, unreactive G101 (magenta) is occluded by positively charged amino acids K89 and K90 in ribosomal protein L24. PDB ID: 4ybb and 8buu were used for *E. coli* and *B. subtilis*, respectively. Images are from PyMol. Panels F and G show the surface.



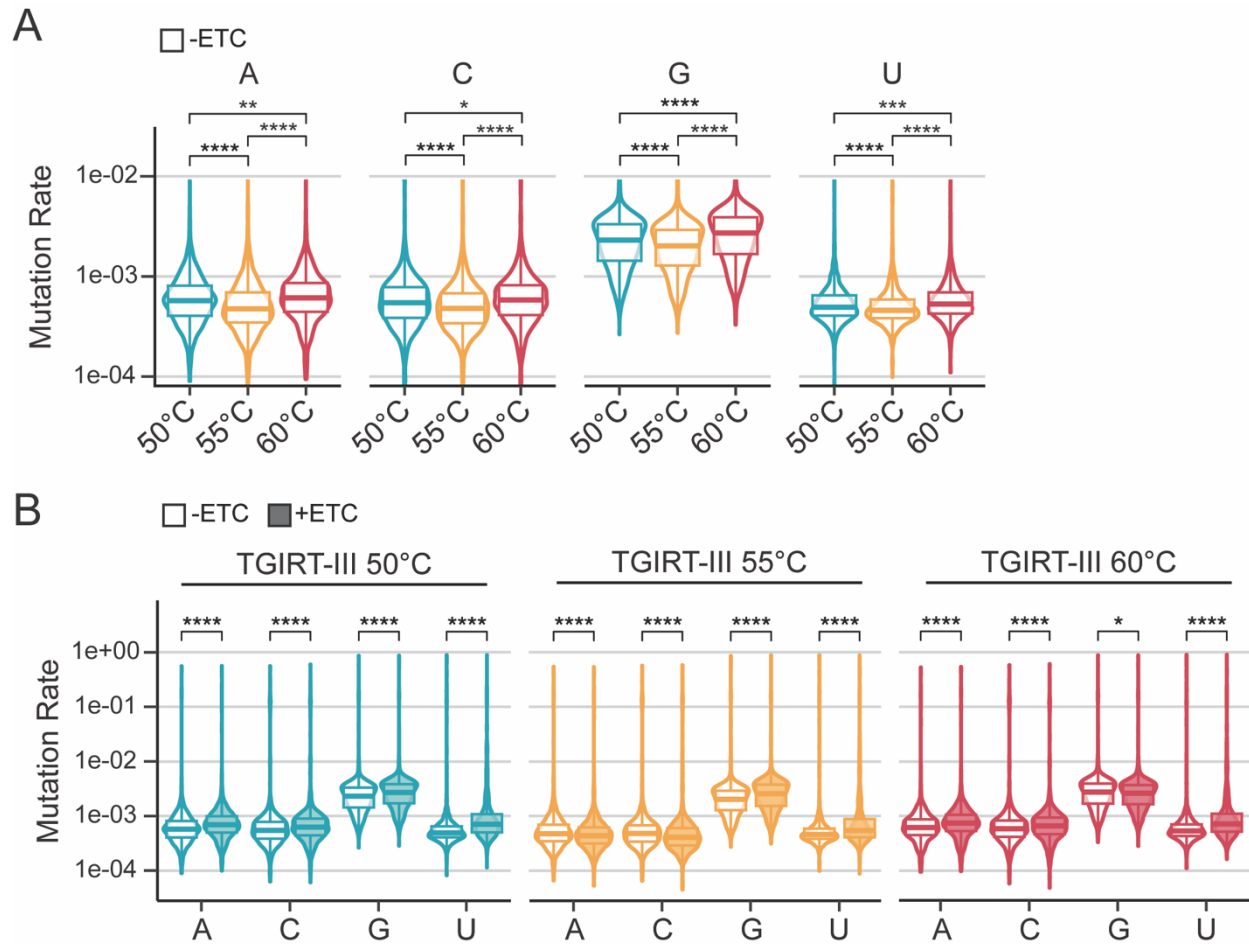
Supplemental Fig. S4: Structural basis for reactive paired (false positive) nucleotides in two base pairs in *B. subtilis* 5S rRNA: (1) Between U72 (magenta) and G100 (yellow), and (2) between G74 (pink) and G98 (yellow). False positive nucleotides are colored magenta, while interacting nucleotides are colored yellow. The polar contact between the N3 of U72 and N2 of G100 is long at 4.4 Å (not shown), suggesting a weakened interaction that leaves the N3 of U72 accessible for modification. Both the N1 and N2 of G74 share a polar contact with the same O6 of G100, suggesting a weakened pairing interaction that supports modification of G74. PDB ID 8buu.



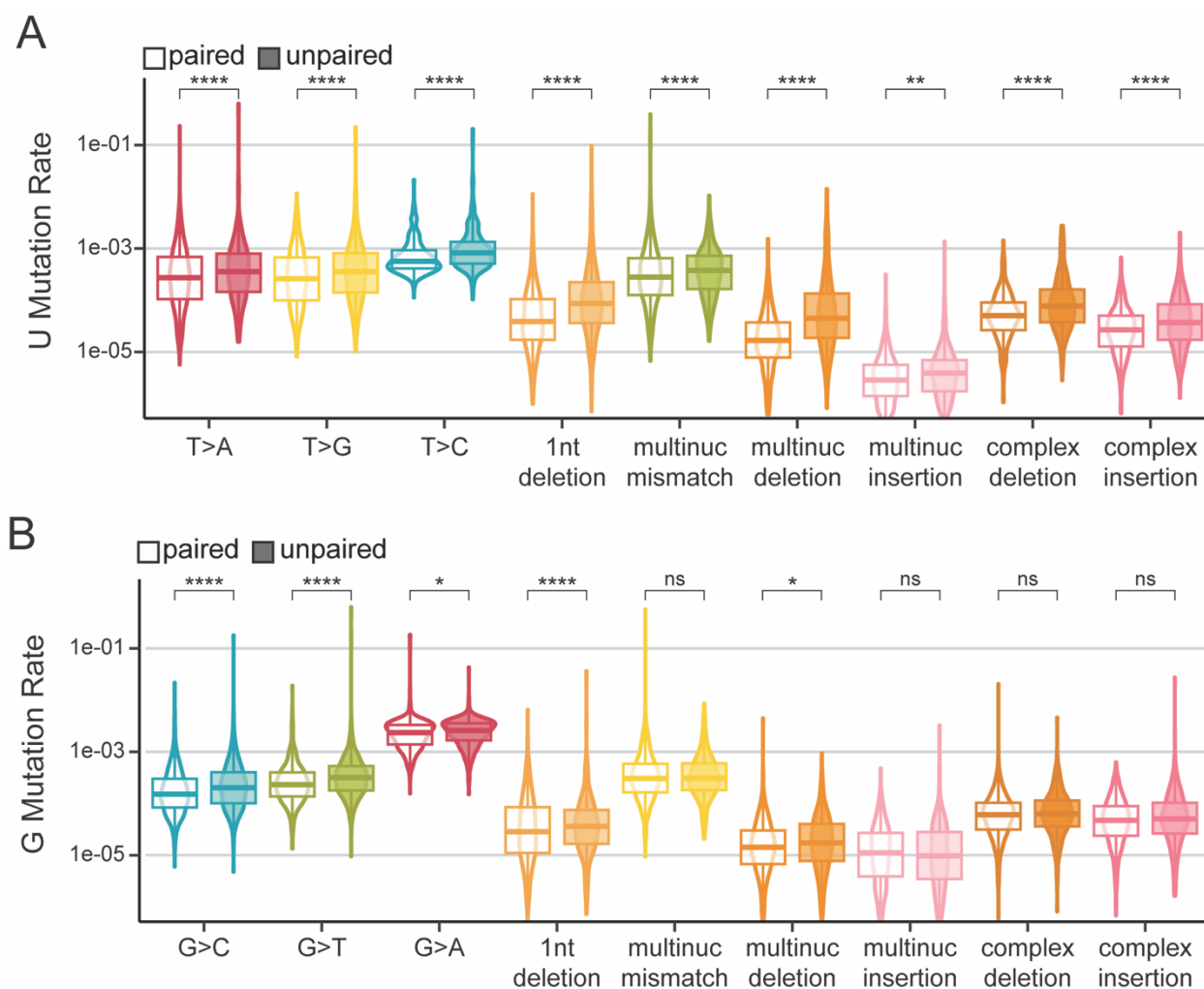
Supplemental Fig. S5: Comparison of mutation rates for RT enzymes for sequencing libraries of *E. coli* 16S rRNA and 23S rRNA probed *in vivo* with ETC. Violin plots show raw mutation rates for ETC-untreated (–ETC) and ETC-treated samples (+ETC), per nucleotide. Significance of Wilcoxon tests are denoted with asterisks, and p-values are provided in Supplemental Table 1.



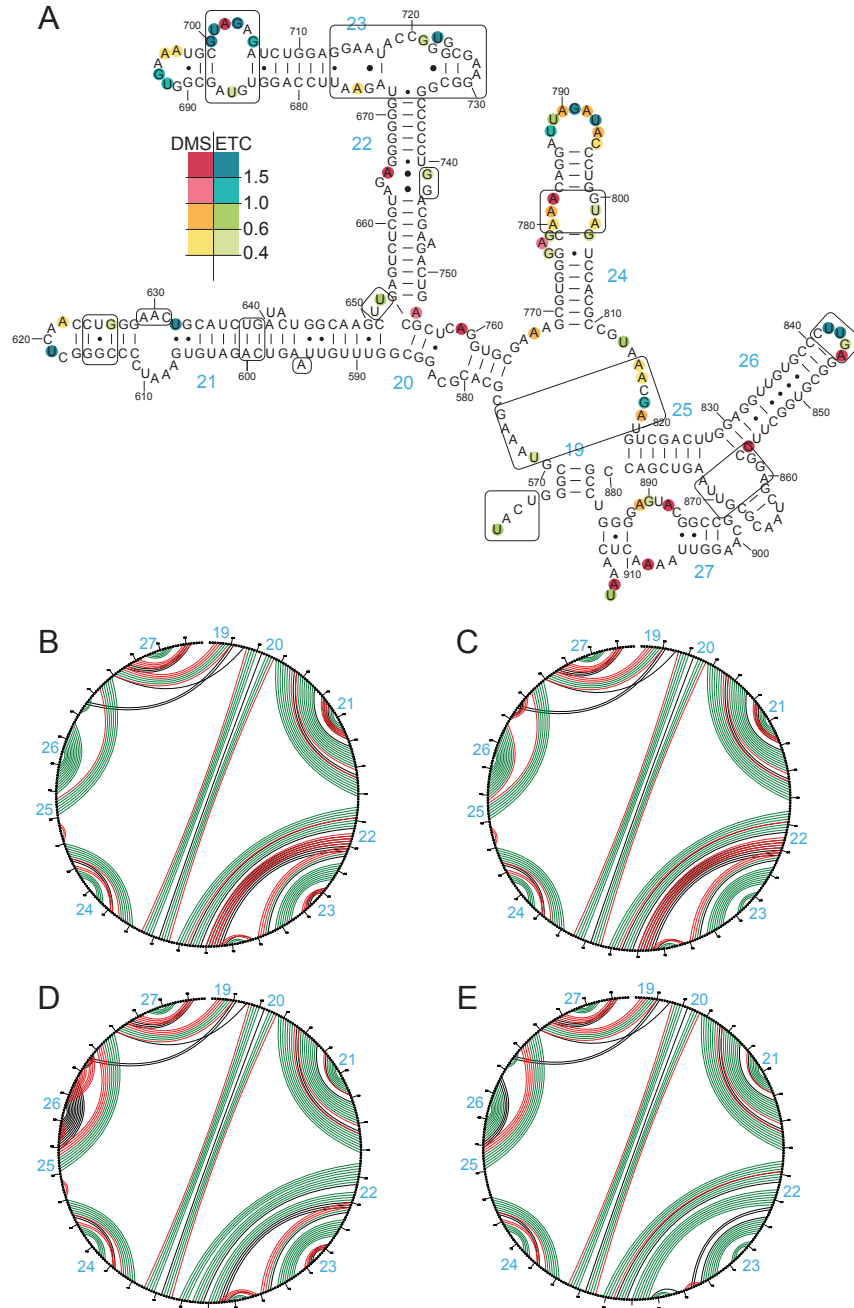
Supplemental Fig. S6: Sequencing library replicate correlation plots. Correlation plots of the raw mutation rates between biological replicates. Each plot displays the Pearson correlation coefficient, R , and the P -value of the correlation.



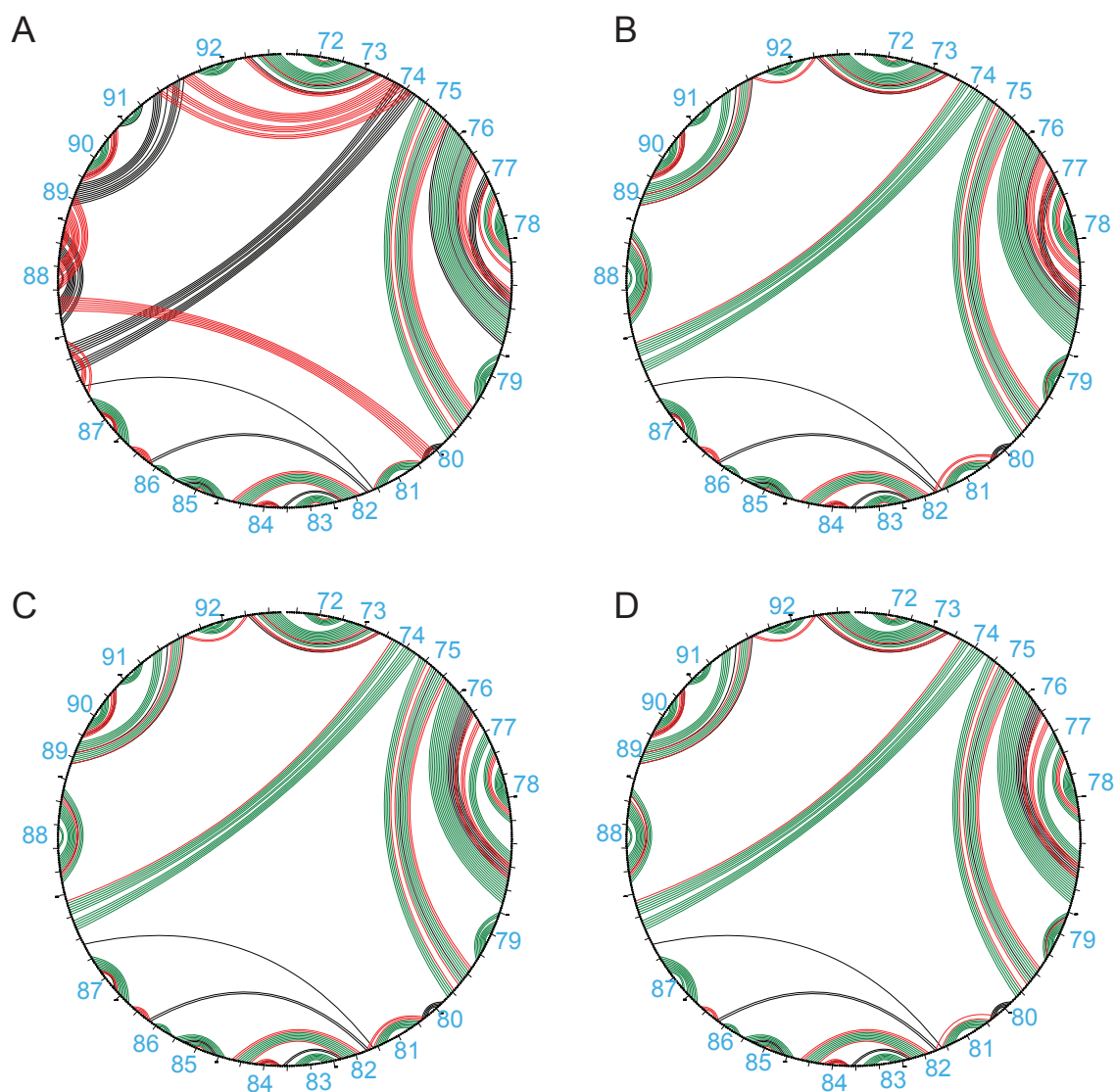
Supplemental Fig. S7: Comparison of RT extension temperatures with TGIRT-III for sequencing libraries of *E. coli* 16S rRNA and 23S rRNA probed *in vivo* with ETC. (A) Violin plots showing raw mutation rates for untreated samples, per nucleotide, as a representation of background signal. Significance of Wilcoxon tests are denoted with asterisks. Precise p-values are provided in Supplemental Table 7. (B) Violin plots showing raw mutation rates for untreated (-ETC) and treated samples (+ETC), per nucleotide. Significance of Wilcoxon tests are denoted with asterisks, and p-values are provided in Supplemental Table 8.



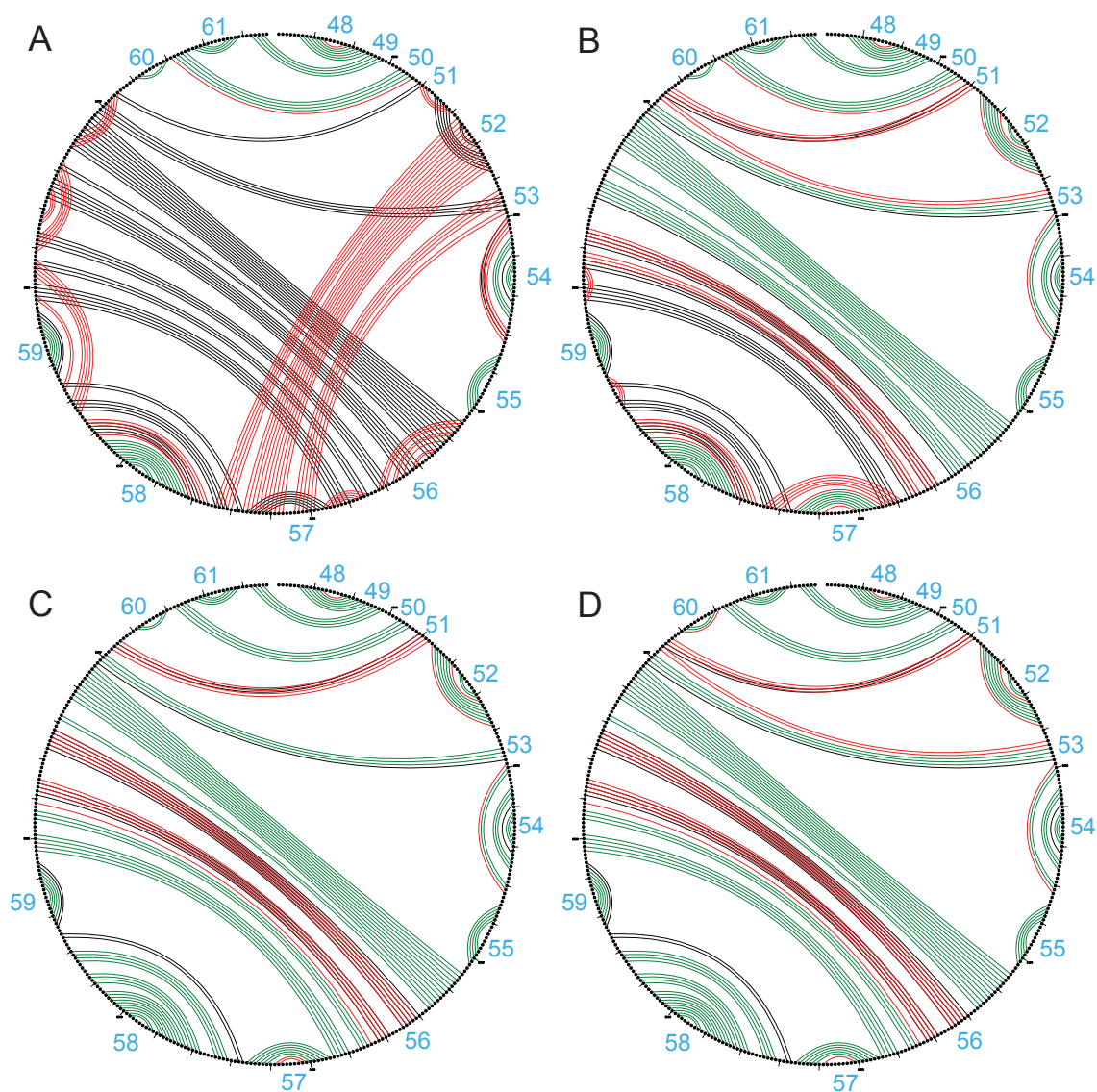
Supplemental Fig. S8: Comparison of mutational signatures for sequencing libraries of *E. coli* 16S rRNA and 23S rRNA probed *in vivo* with ETC. Violin plots showing background-subtracted mutation rates for paired (unfilled) and unpaired (filled) nucleotides at each type of mutation at (A) U and (B) G. Medians are provided in Supplemental Tables (A) 12 and (B) 14. Significance of Wilcox tests are denoted with asterisks, and p-values are provided in Supplemental Tables (A) 13 and (B) 15.



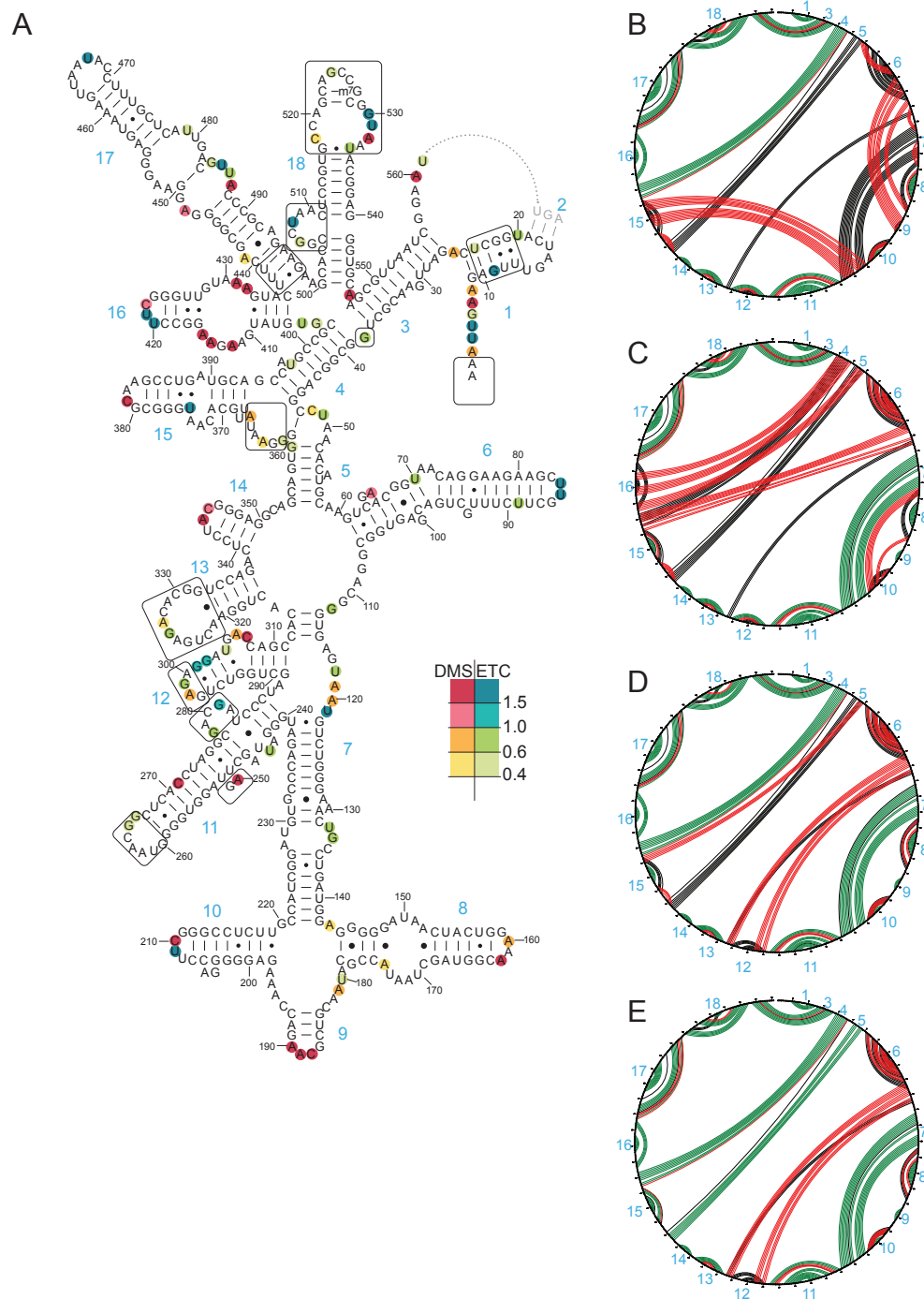
Supplemental Fig. S9: Folding of the *E. coli* 16S central domain. (A) DMS reactivity at As and Cs and ETC reactivity at Gs and Us mapped onto known structure. Reactivity values are provided in the legend. (B-E) Circle plots comparing the known structure to the predicted structure using (B) no pseudo-free energy restraints, (C) ETC reactivities at Gs and Us as restraints, (D) DMS reactivities at As and Cs as restraints, and (E) combined ETC and DMS reactivity as restraints. Correct base pairs are green, incorrect base pairs are red, and unpredicted base pairs are black.



Supplemental Fig. S10: Folding of the *E. coli* 23S domain V. Circle plots comparing the known structure to the predicted structure using (A) no pseudo-free energy restraints, (B) ETC reactivities at Gs and Us as restraints, (C) DMS reactivities at As and Cs as restraints, and (D) combined ETC and DMS reactivities as restraints. Correct base pairs are green, incorrect base pairs are red, and unpredicted base pairs are black.



Supplemental Fig. S11: Folding of the *E. coli* 23S domain III. Circle plots comparing the known structure to the predicted structure using (A) no pseudo-free energy restraints, (B) ETC reactivities at Gs and Us as restraints, (C) DMS reactivities at As and Cs as restraints, and (D) combined ETC and DMS reactivities as restraints. Correct base pairs are green, incorrect base pairs are red, and unpredicted base pairs are black.



Supplemental Fig. S12: Folding of the *E. coli* 16S 5' domain. (A) DMS reactivity at As and Cs and ETC reactivity at Gs and Us mapped onto the known structure with reactivity values indicated by the legend and black boxes indicating protein interactions. (B-E) Circle plots comparing the known structure to the predicted structure using B) no pseudo-free energy restraints, (C) ETC reactivities at Gs and Us as restraints, (D) DMS reactivities at As and Cs as restraints, and (E) combined ETC and DMS reactivities as restraints. Correct base pairs are green, incorrect base pairs are red, and unpredicted base pairs are black.