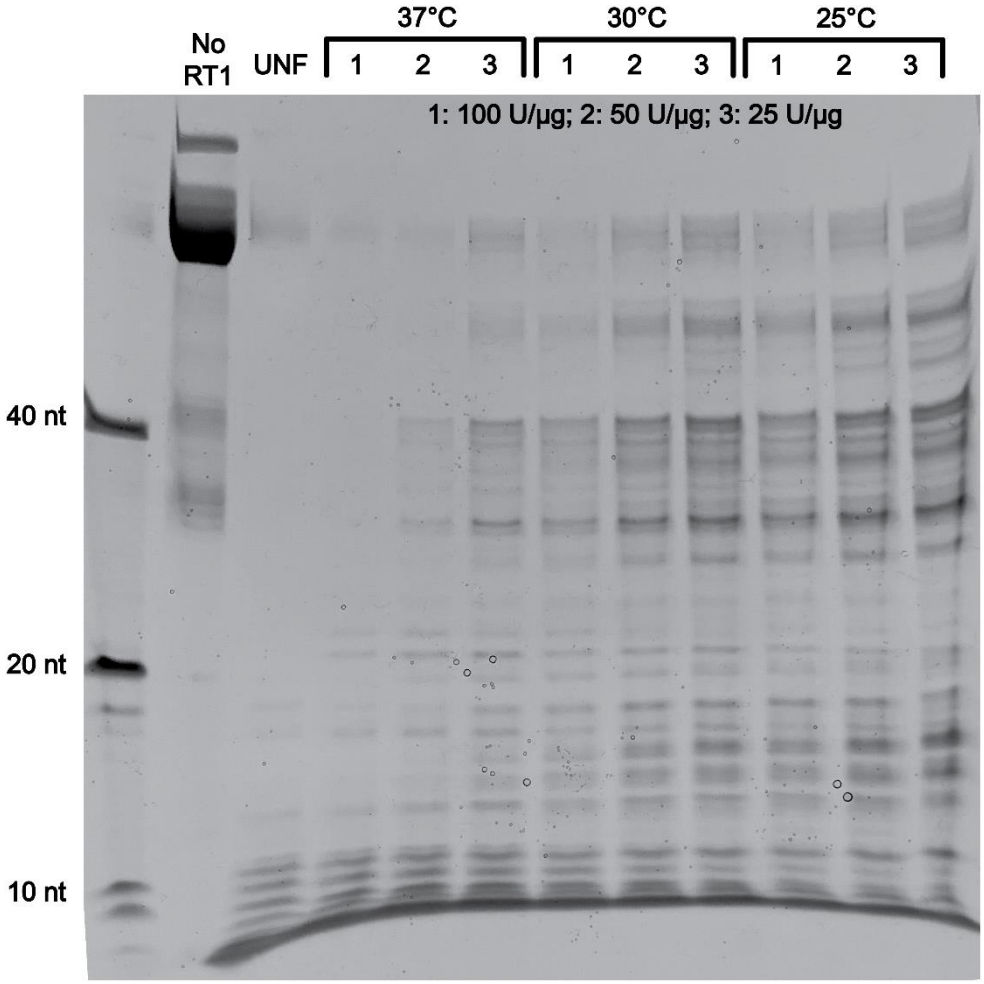
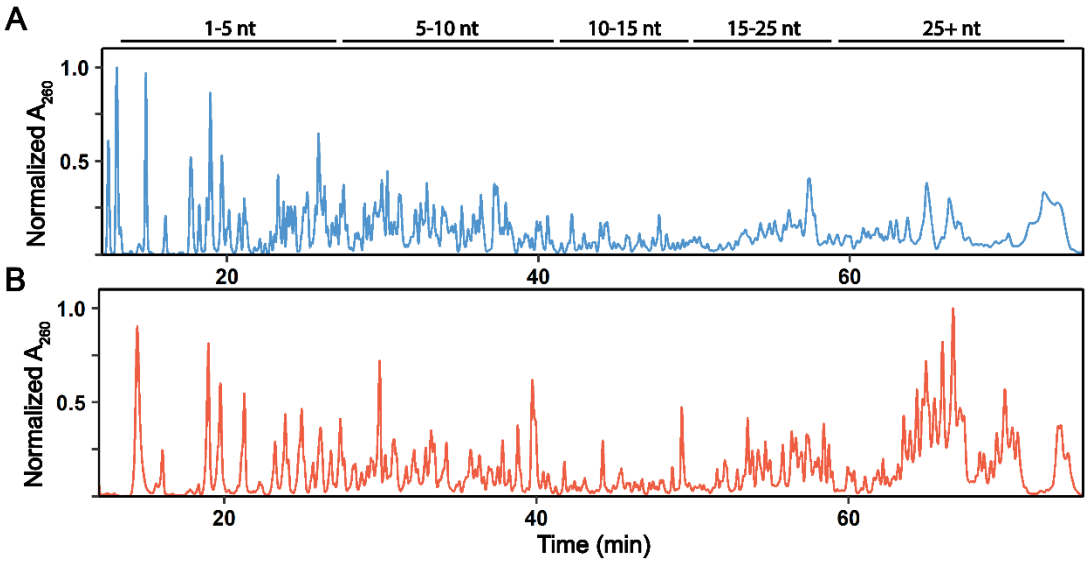
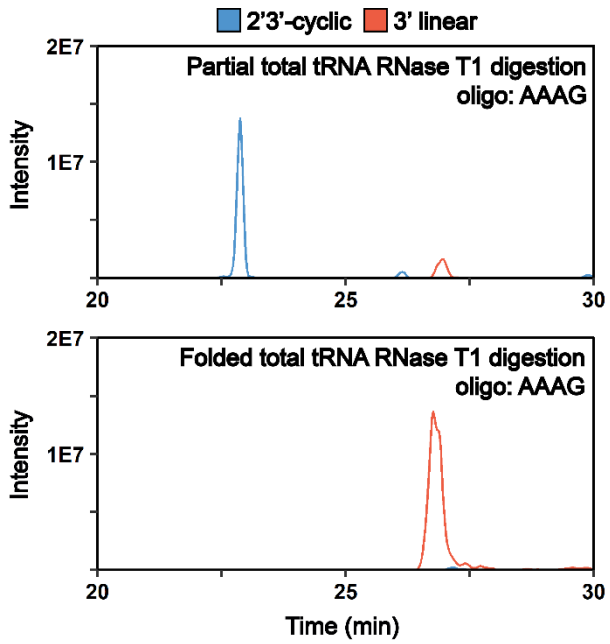


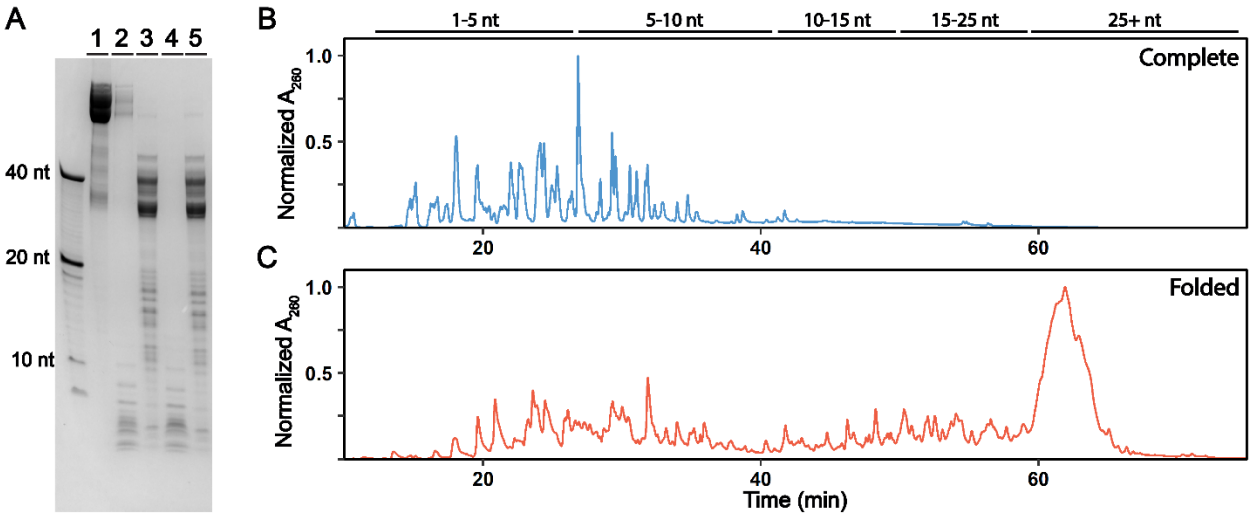
Supplemental Figure S1: Temperature dependence of tRNA structure inhibition of RNase T1 digestion. *S. cerevisiae* total tRNA was either incubated with no RNase T1 (No RT1), digested with 100 U/µg RNase T1 at 37℃ (UNF), or folded prior to digestion with RNase T1. The folded digestions were performed at either 37℃, 30℃, or 25℃ at 100 U/µg RNase T1 (condition 1), 50 U/µg RNase T1 (condition 2), or 25 U/µg RNase T1 (condition 3).



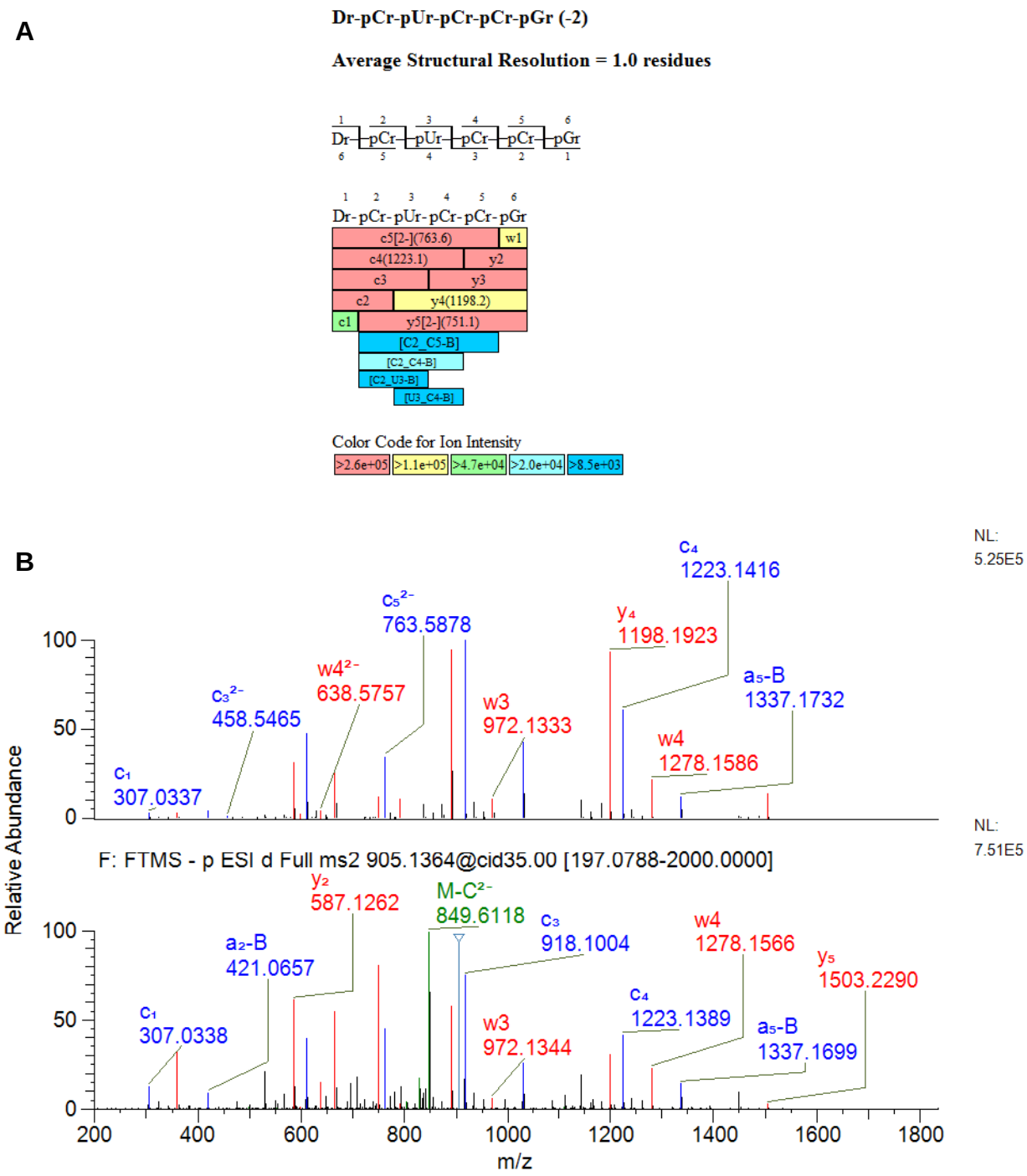
Supplemental Figure S2: Folded total tRNA RNase T1 digestions produces more unique digestion products than partial total tRNA digestions. (A) LC-UV analysis of *S. cerevisiae* total tRNA partial digestion with 40 mU/μg RNase T1 for 30 min at 37℃. **(B)** LC-UV analysis of folded *S. cerevisiae* total tRNA digestion with 25 U/μg RNase T1 for 1 hr at 25℃.



Supplemental Figure S3: Folded total tRNA RNase T1 digestions produce only 3' linear phosphate products. Extracted ion chromatograms of AAAG oligonucleotide digestion product detected in *S. cerevisiae* total tRNA digestions. Partial digestions produce a mixture of 2'3'-cyclic (blue) and 3' linear (red) phosphates, while folded tRNA digestions only produce 3' linear phosphates.

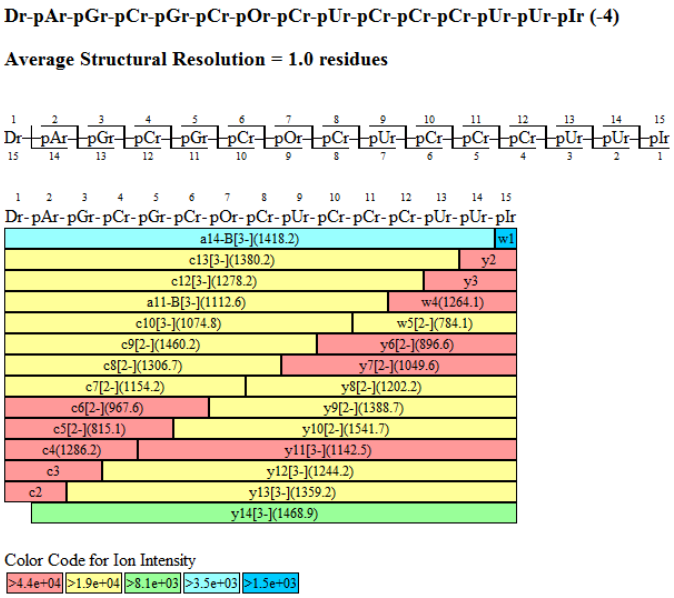


Supplemental Figure S4: tRNA secondary structure inhibits digestion by single stranded specific RNase A. (A) Denaturing urea-PAGE gel electrophoresis displaying the oligonucleotide digestion products produced during complete and folded RNase A digestions with 25 ng/μg RNase A at 25℃ with the following digestion conditions: Lane 1: undigested *S. cerevisiae* total tRNA; lane 2: complete digestion in NH_4OAc; lane 3: digestion in NH_4OAc + 20 mM $MgCl_2$; lane 4: MES + KCl; lane 5: heat denatured tRNA in MES + KCl + $MgCl_2$. **(B)** LC-UV of total tRNA complete RNase A digestion (lane 2 of panel A). **(C)** LC-UV of folded total tRNA RNase A digestion (lane 5 of panel A).

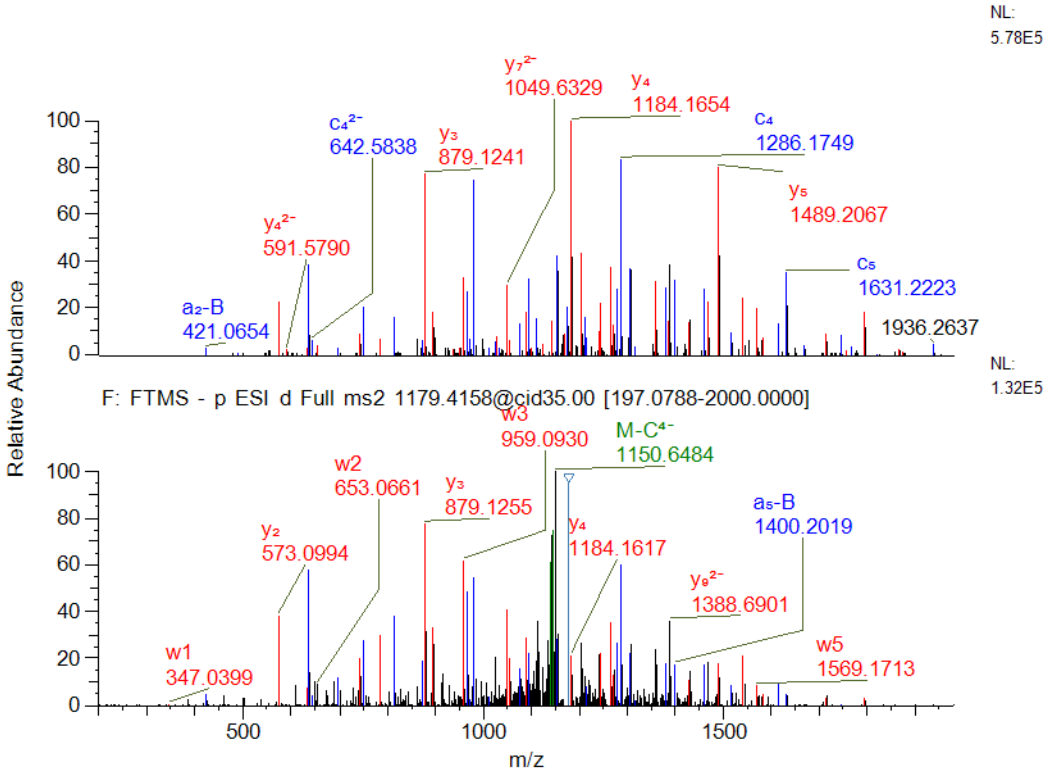


Supplemental Figure S5: MS/MS sequencing of DCUCCG oligonucleotide digestion product in *S. cerevisiae* tRNA$^{Ala(AGC)}$. (A) Sequence coverage map of oligonucleotide digestion product displaying the MS/MS fragments detected. **(B)** The MS/MS scan of 905.1364 m/z ion (bottom panel) corresponds well to theoretical MS/MS spectra of DCUCCG oligonucleotide.

A

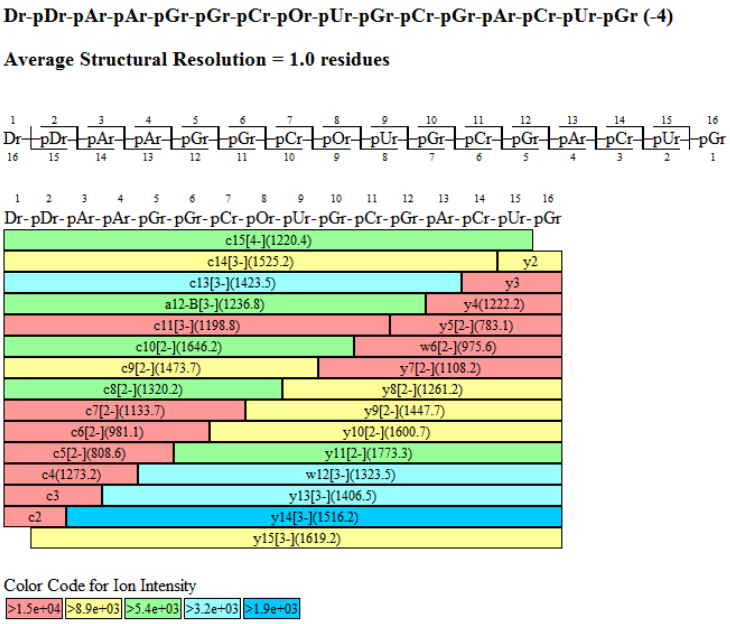


B

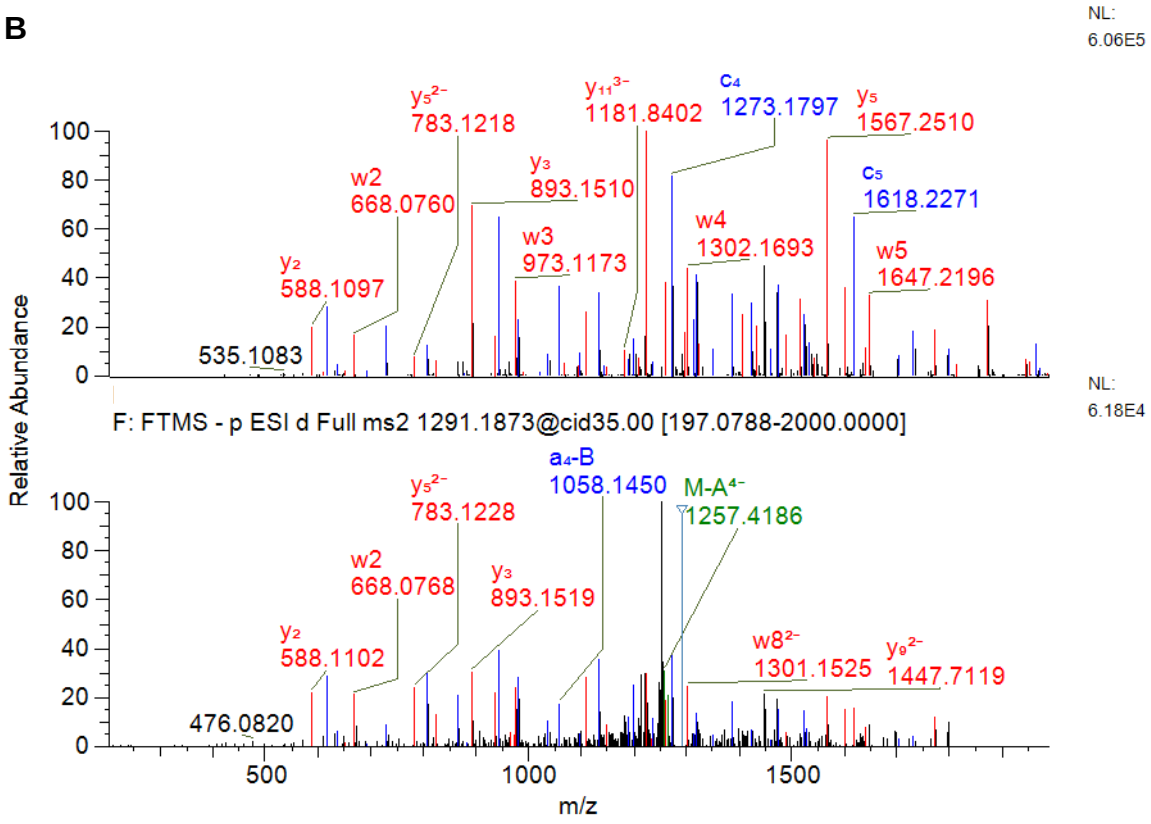


Supplemental Figure S6: MS/MS sequencing of DAGCGCm$^2_2$GCUCCCUUI oligonucleotide digestion product in *S. cerevisiae* tRNA$^{Ala(AGC)}$. (A) Sequence coverage map of oligonucleotide digestion product displaying the MS/MS fragments detected. **(B)** The MS/MS scan of 1179.4158 m/z ion (bottom panel) corresponds well to theoretical MS/MS spectra of DAGCGCm$^2_2$GCUCCCUUI oligonucleotide.

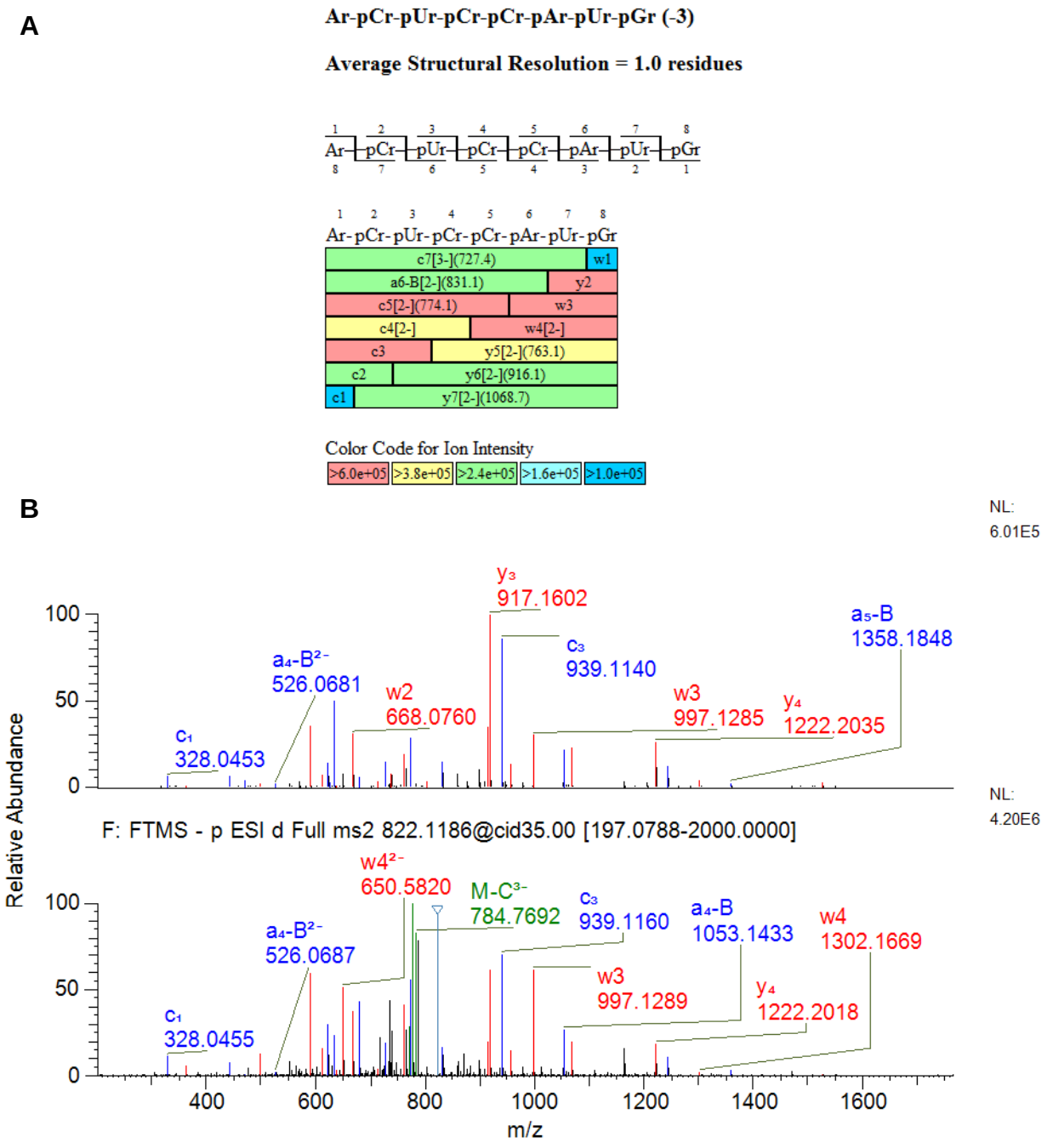
A



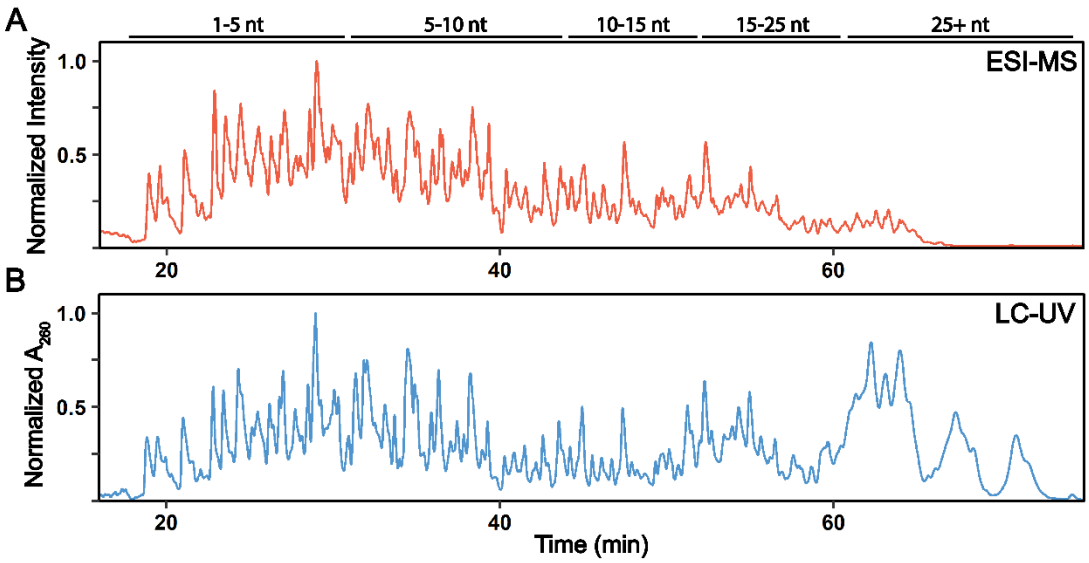
B



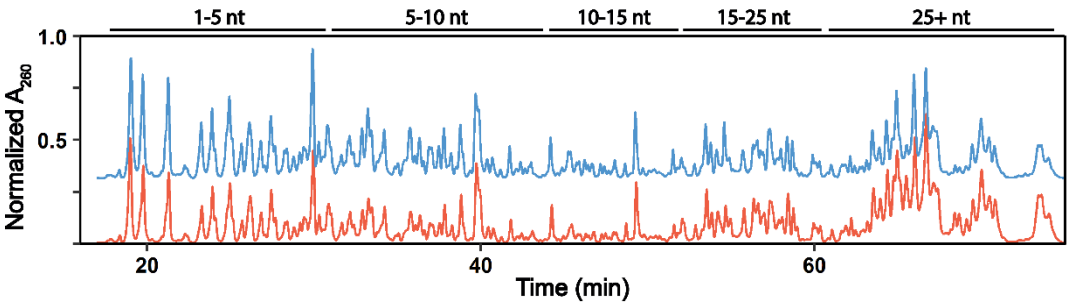
Supplemental Figure S7: MS/MS sequencing of DDAAGGCm$^2_2$GUGCGACUG oligonucleotide digestion product in *S. cerevisiae* tRNA$^{Asn(GUU)}$. (A) Sequence coverage map of oligonucleotide digestion product displaying the MS/MS fragments detected. **(B)** The MS/MS scan of 1291.1873 m/z ion (bottom panel) corresponds well to theoretical MS/MS spectra of DDAAGGCm22GUGCGACUG oligonucleotide.



Supplemental Figure S8: MS/MS sequencing of ACUCCAUG oligonucleotide digestion product in *S. cerevisiae* tRNA$^{Asn(GUU)}$. (A) Sequence coverage map of oligonucleotide digestion product displaying the MS/MS fragments detected. **(B)** The MS/MS scan of 822.1186 m/z ion (bottom panel) corresponds well to theoretical MS/MS spectra of ACUCCAUG oligonucleotide.



Supplemental Figure S9: ESI-MS and LC-UV signal intensity comparison of folded total tRNA digestion. (A) ESI-MS total ion chromatogram and **(B)** LC-UV chromatogram of folded *S. cerevisiae* total tRNA RNase T1 digestions displaying the discrepancies in signal distribution for longer oligonucleotide digestion products. LC-UV detects a higher abundance of longer oligonucleotide digestion products than ESI-MS, likely due to poor ESI ionization efficiencies of longer oligonucleotides.



Supplemental Figure S10: Folded total tRNA RNase digestions are reproducible. Overlaid LC-UV chromatograms of two biological replicate samples. Each total tRNA replicate was folded and treated with RNase T1. Digestions were performed on two separate days. The striking similarity of the chromatograms demonstrates the highly reproducible nature of these digestions.