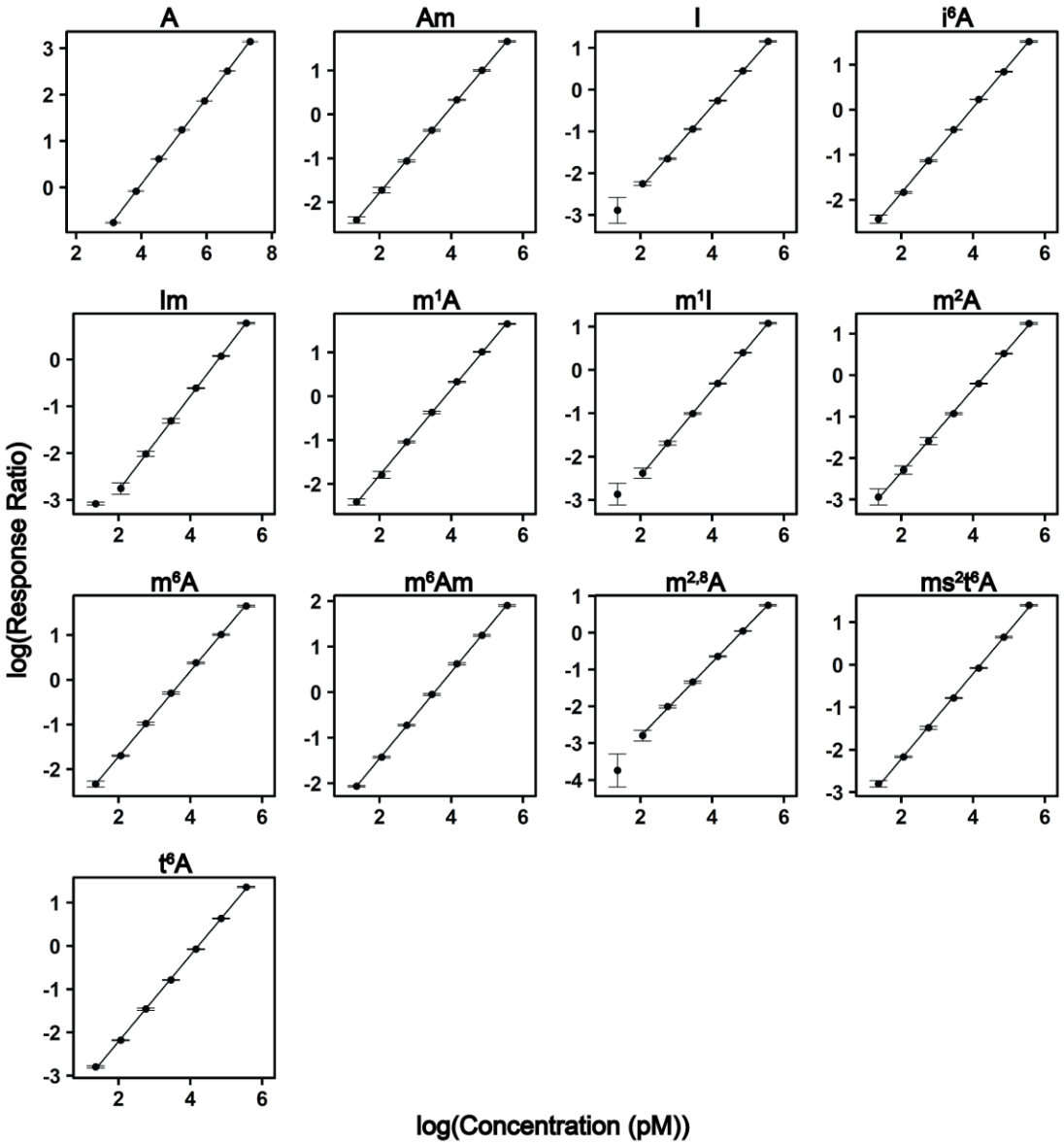
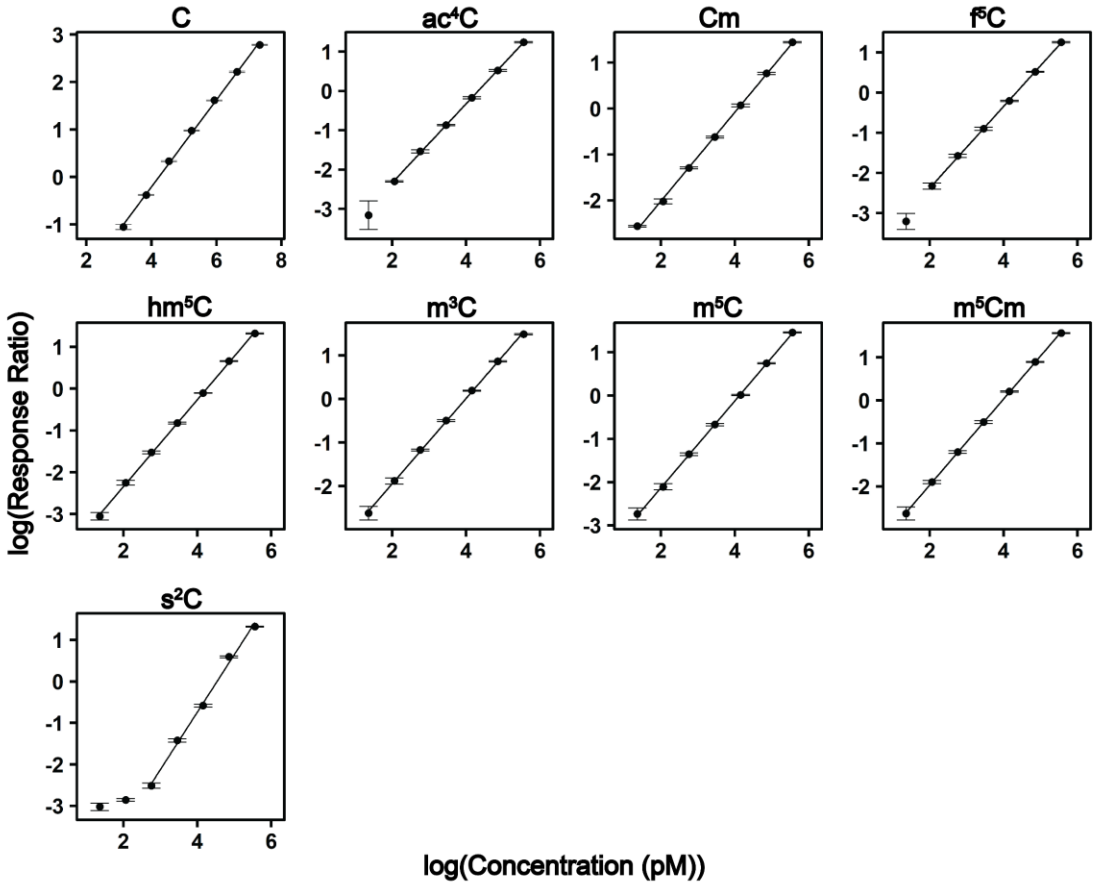
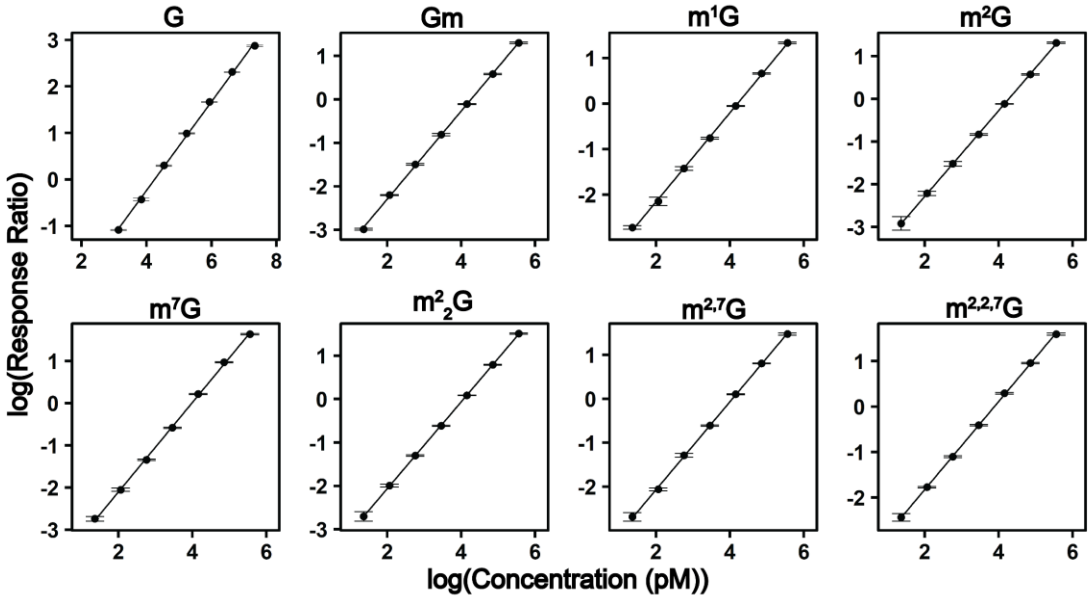


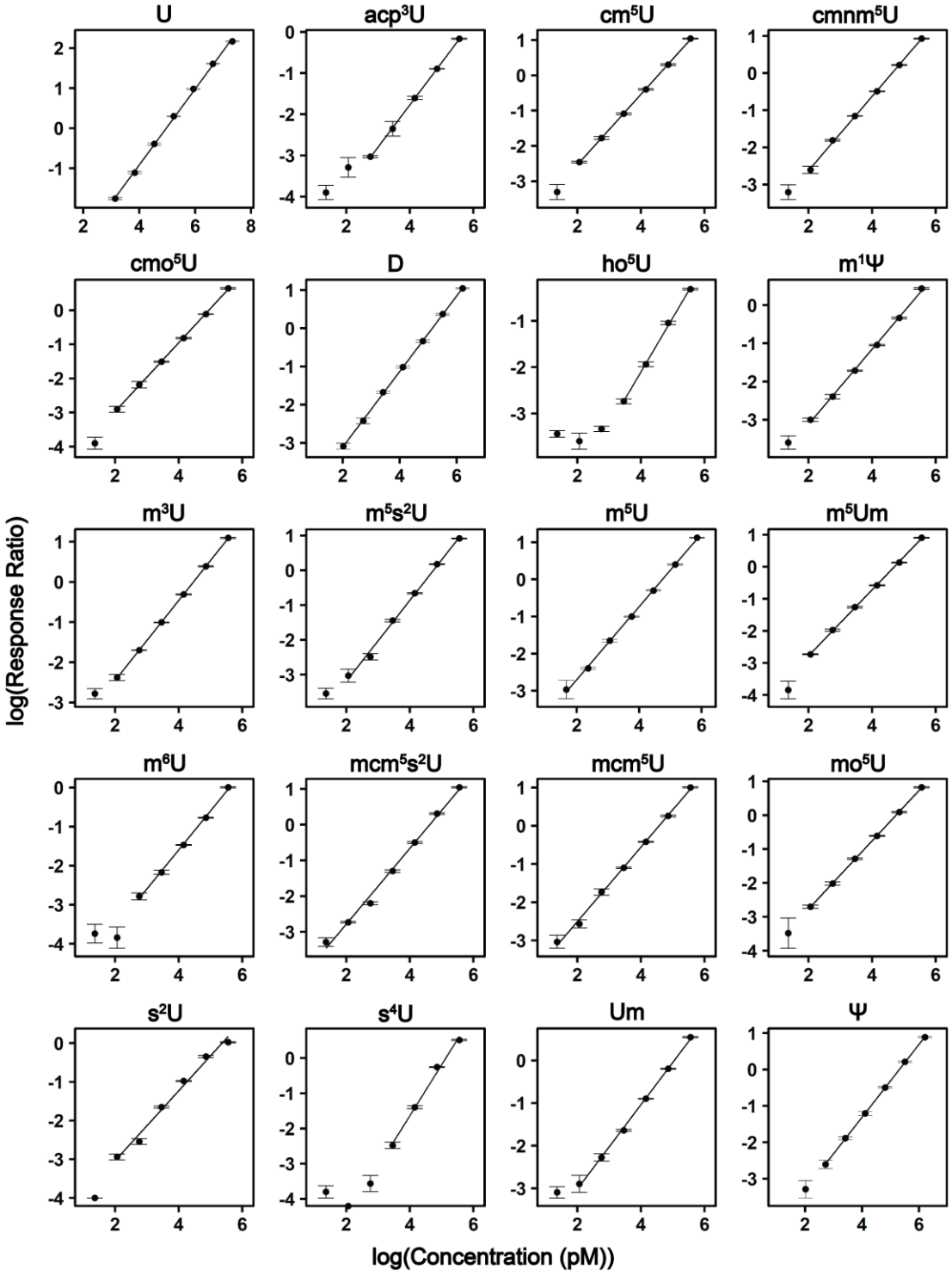
Supplemental Figure S1: Calibration curves used to quantify adenosine modification concentrations. Calibration curves of adenosine ribonucleoside modifications plotted in log(response ratio) vs. log(concentration (pM)). The linear regression, limit of detection, and R^2 are displayed in **Supplemental Table S1.**



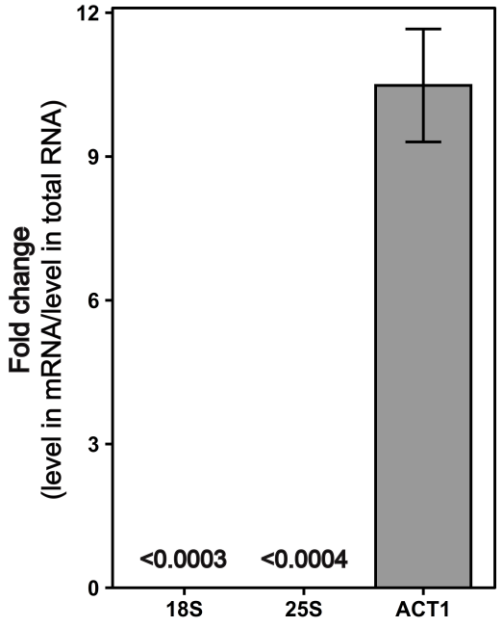
Supplemental Figure S2: Calibration curves used to quantify cytidine modification concentrations. Calibration curves of cytidine ribonucleoside modifications plotted in log(response ratio) vs. log(concentration (pM)). The linear regression, limit of detection, and R^2 are displayed in **Supplemental Table S1.**



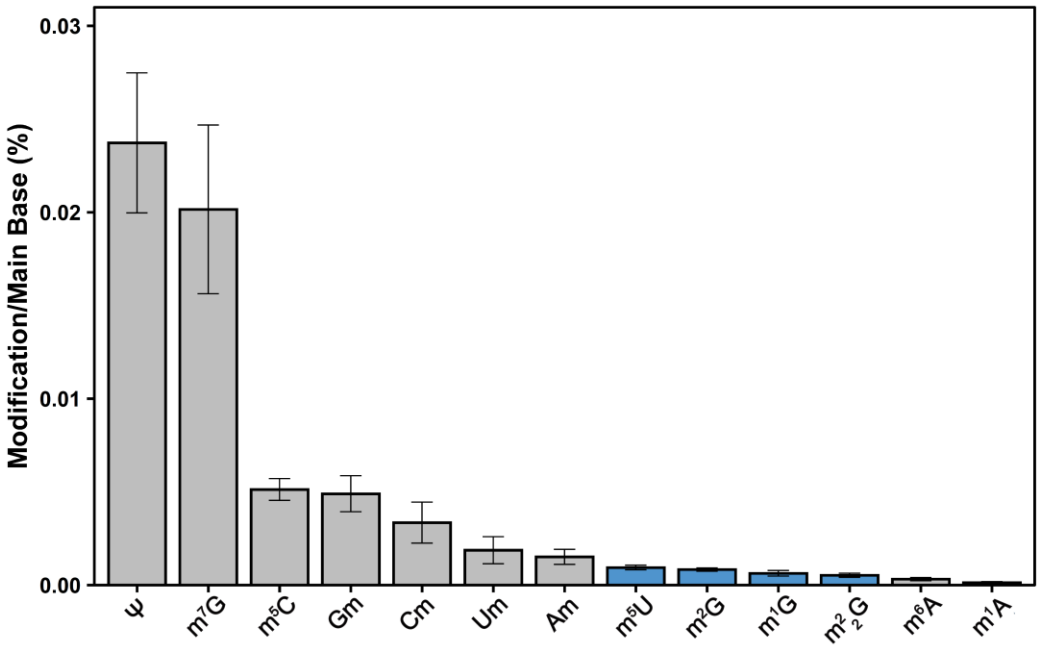
Supplemental Figure S3: Calibration curves used to quantify guanosine modification concentrations. Calibration curves of guanosine ribonucleoside modifications plotted in log(response ratio) vs. log(concentration (pM)). The linear regression, limit of detection, and R^2 are displayed in **Supplemental Table S1.**



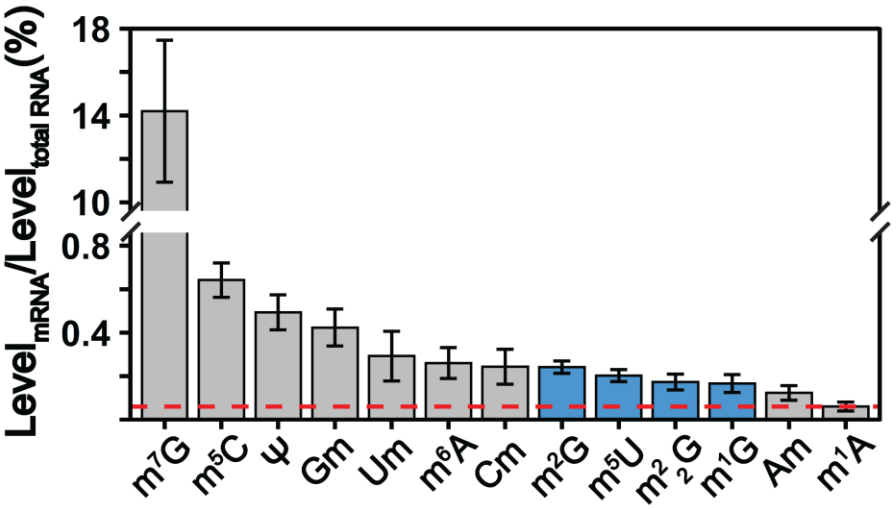
Supplemental Figure S4: Calibration curves used to quantify uridine modification concentrations. Calibration curves of uridine ribonucleoside modifications plotted in log(response ratio) vs. log(concentration (pM)). The linear regression, limit of detection, and R^2 are displayed in **Supplemental Table S1.**



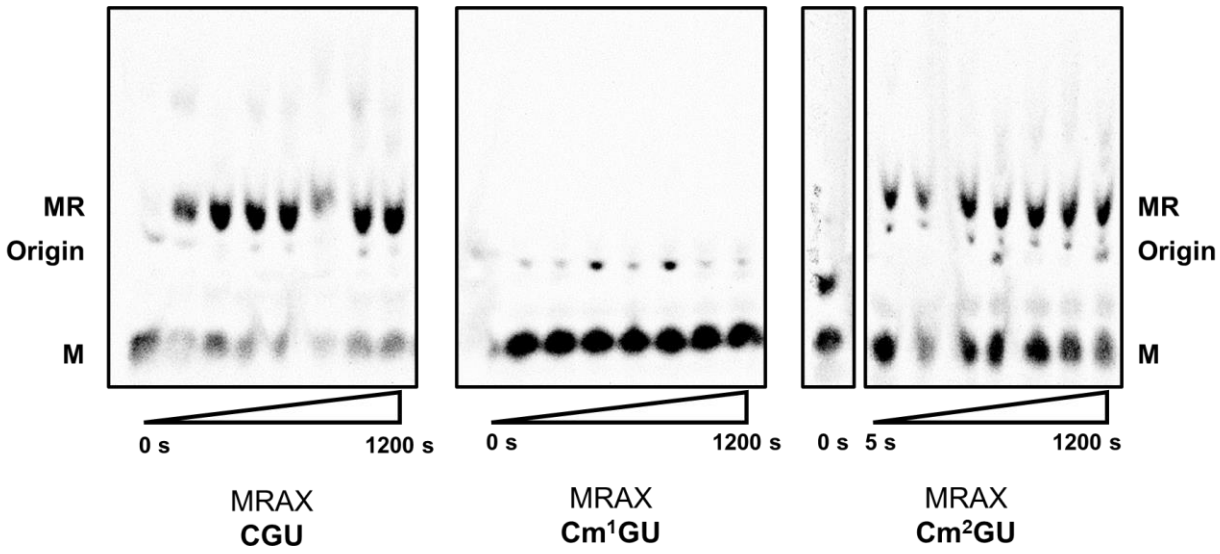
Supplemental Figure S5: Ribosomal RNAs are depleted in three-stage purified mRNA. qRT-PCR demonstrates that the 18S and 25S rRNAs are depleted by greater than 3000-fold in the purified mRNA. Contrarily, ACT1 is enriched by greater than 10-fold. This data in addition to the Bioanalyzer electropherograms, RNA-seq, and LC-MS/MS proves that our three-stage purified mRNA is highly pure.



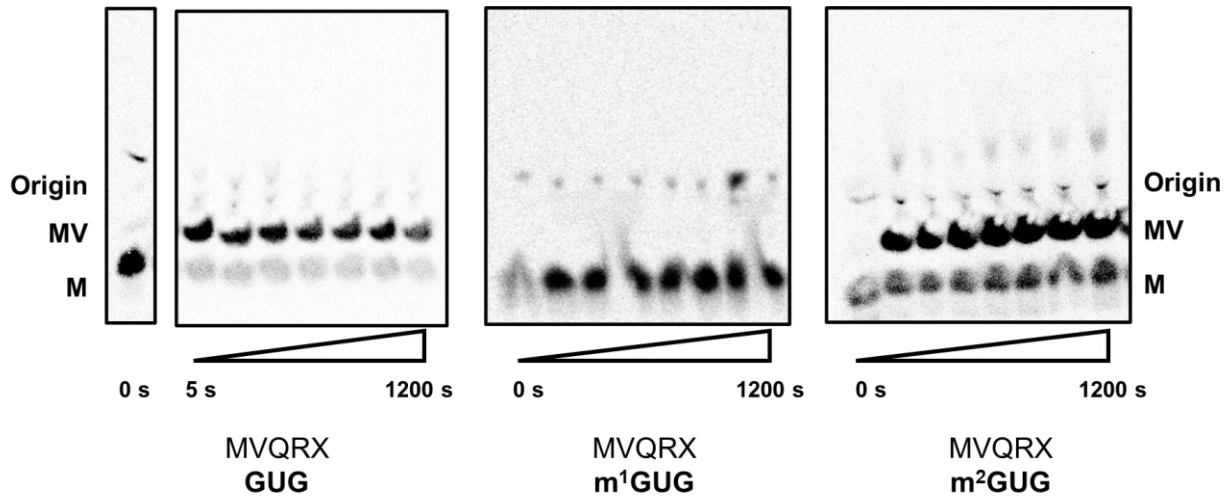
Supplemental Figure S6: Ribonucleoside modification abundance in the three-stage purified mRNA. The ribonucleoside abundance is represented as modification/main base% (i.e., m^7G/G%) where pseudouridine was the most abundant modification detected. All modifications detected were previously detected in purified mRNA besides for the three methylated guanosine modifications displayed in blue (m^1G, m^2G, and $m^2{}_2G$). Our improvements regarding LC-MS/MS sensitivity and mRNA purity enables us to confidently claim these modifications exist with *S. cerevisiae* mRNA.



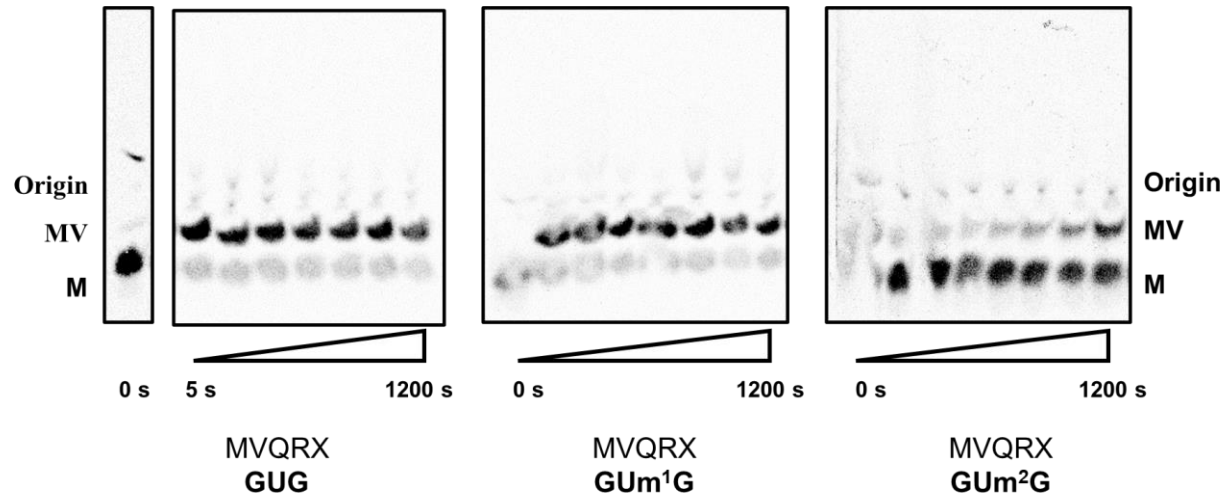
Supplemental Figure S7: RNA modification percent retention in purified mRNA. The percent retention of modifications found in mRNA compared to the levels in totalRNA (mod/main% in mRNA compared to mod/main% in total RNA). m^1G, m^2G, $m^2{}_2G$, and m^5U are only present in *S. cerevisiae* tRNA; thus, we reasoned that they would be retained at a higher percentage than other highly abundant tRNA modifications if they are present in mRNA. Dihydrouridine, which is the most abundant non-mRNA modification in tRNA, was not detected in our purified mRNA samples. If dihydrouridine existed at levels just below our limit of detection (530 amol), the maximum retention of solely tRNA modifications would be 0.06% ($D_{mRNA}/D_{totalRNA}$ (%); red dashed line). The four new mRNA modifications we detect, along with all other known mRNA modifications, are retained at greater extents which proves these modifications exist in *S. cerevisiae* mRNA. The error bars are the standard deviation of the percent retention.



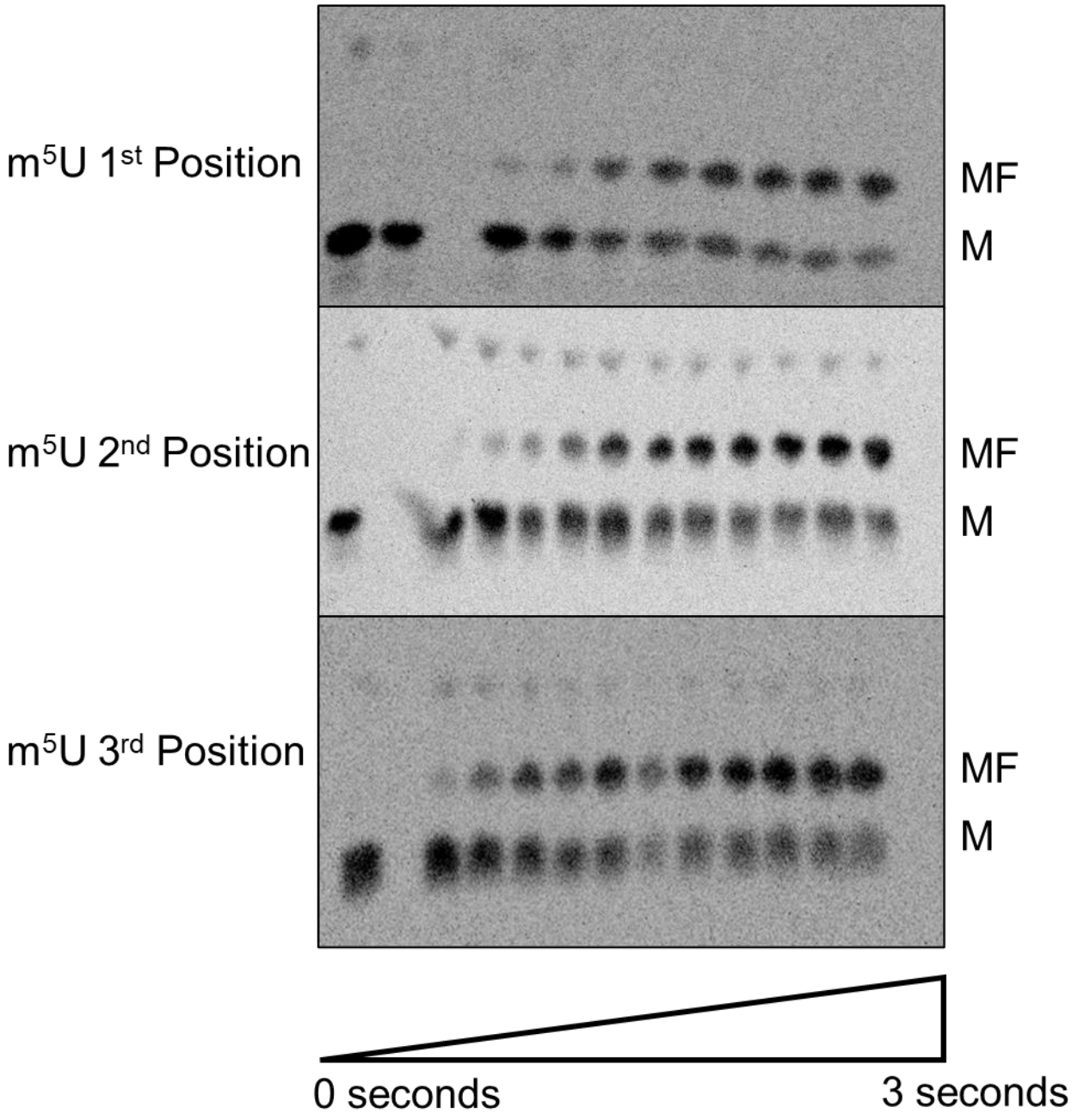
Supplemental Figure S8: Electrophoretic TLC displaying the translation products of CGU, Cm^1GU, and Cm^2GU codons in the presence of arginine tRNA (ArgTC), forming MR dipeptide over the span of 1200 seconds.



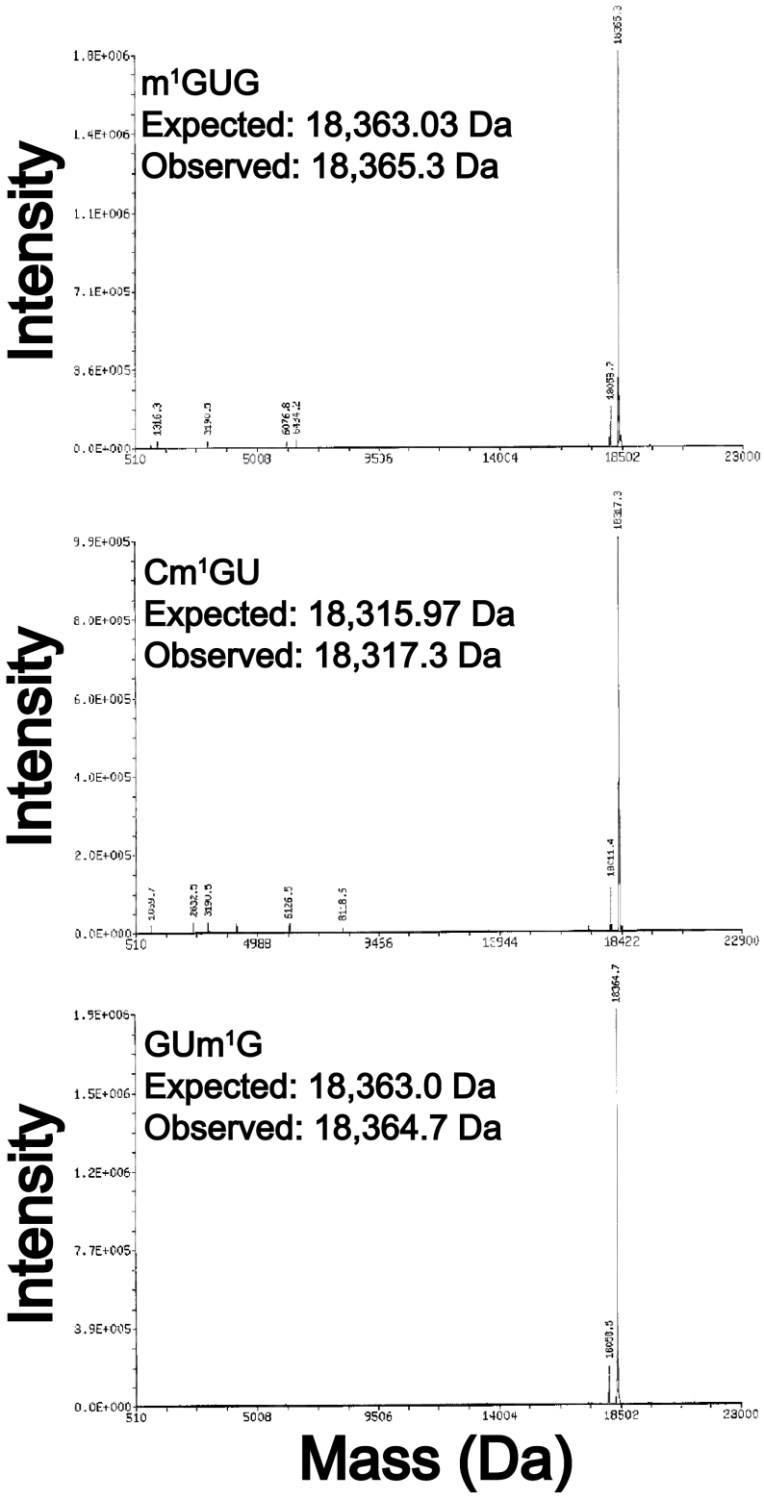
Supplemental Figure S9: Electrophoretic TLC displaying the translation products of GUG, m^1GUG, and m^2GUG codons in the presence of valine tRNA (ValTC), forming MV dipeptide over the span of 1200 seconds.



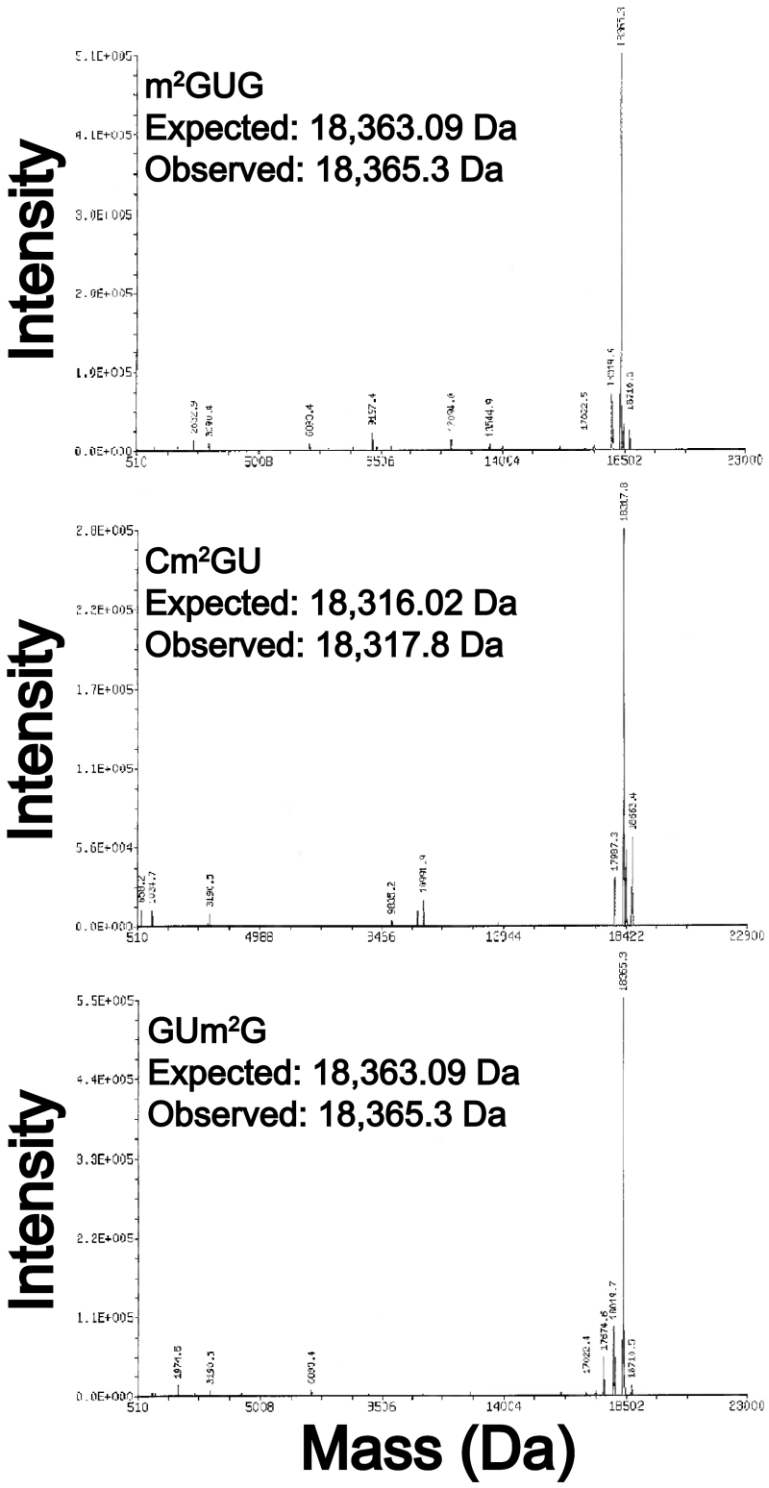
Supplemental Figure S10: Electrophoretic TLC displaying the translation products of GUG, GUm^1G, and GUm^2G codons in the presence of valine tRNA (ValTC), forming MV dipeptide over the span of 1200 seconds.



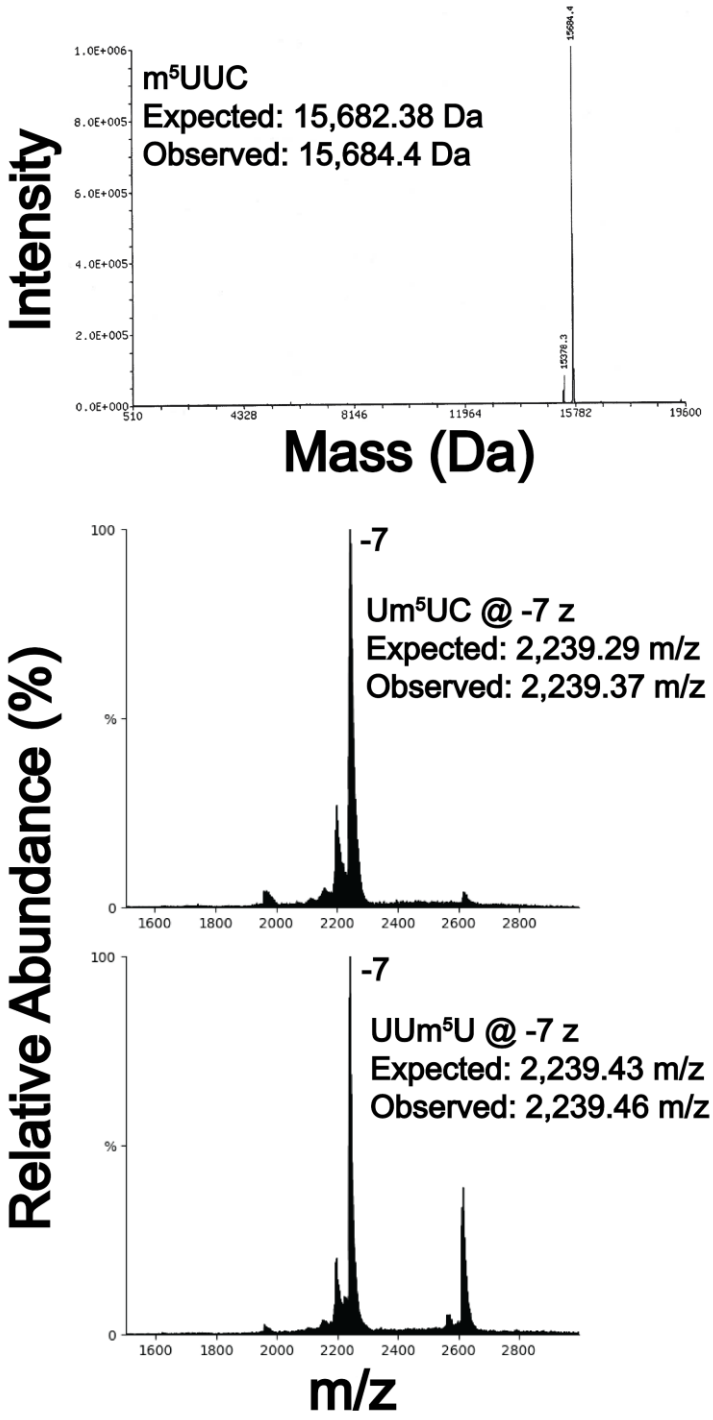
Supplemental Figure S11: Electrophoretic TLC displaying the translation products of m^5U messages in the presence of phenylalanine tRNA (PheTC), forming MF dipeptide over the span of 3 seconds.



Supplemental Figure S12: Deconvoluted ESI-MS spectra of modified oligonucleotides provided by Dharmacon to confirm purity. The expected and observed masses of the m^1GUG, Cm^1GU, and GUm^1G modified codon oligonucleotides are found in the top, middle, and bottom panels, respectively. Minor n-1 oligonucleotides products were detected, but they would not affect the *in vitro* translation assays because the nucleotide loss occurs in the non-coded region of the purchased mRNA transcript.



Supplemental Figure S13: Deconvoluted ESI-MS spectra of modified oligonucleotides provided by Dharmacon to confirm purity. The expected and observed masses of the m^2GUG, Cm^2GU, and GUm^2G modified codon oligonucleotides are found in the top, middle, and bottom panels, respectively. Minor n-1 oligonucleotides products were detected, but they would not affect the *in vitro* translation assays because the nucleotide loss occurs in the non-coded region of the purchased mRNA transcript.



Supplemental Figure S14: Deconvoluted ESI-MS spectra of m^{5}UUC modified codon oligonucleotides provided by Dharmacon to confirm purity (top panel). Full scan spectra of Um^{5}UC (middle) and UUm^{5}U (bottom) modified codon oligonucleotide. The corresponding expected and observed mass (Da) or mass-to-charge (m/z) is displayed for each spectrum. Minor n-1 oligonucleotides products were detected, but they would not affect the *in vitro* translation assays because the nucleotide loss occurs in the non-coded region of the purchased mRNA transcript.

$$\frac{25\ pg/\mu L}{3000\ pg/\mu L}\ x\ 100\% = 0.8\%$$

Supplemental Calculation S1: The Bioanalyzer RNA 6000 pico assay provides an LOD of 25 pg/uL for a single RNA[1]; thus, the maximum theoretical tRNA or rRNA contamination would be 0.8% if it was just below our detection limit (3000 pg/uL sample analyzed).

$$\frac{(DHU\ LOD)(Avg\ tRNA\ MW)}{(Avg\ DHU\ per\ tRNA)(mRNA\ digested)} * 100\% = maximum\ tRNA\ contamination\ (\%)$$

$$\frac{(5.30E-16\ mol)(28{,}000\ g/mol)}{(3\ DHU\ per\ tRNA)(200E-9\ g)} * 100\% = 0.002\%$$

Supplemental Calculation S1: The maximum tRNA contamination can be estimated based on the limit of detection for DHU. Since we know the quantity of mRNA digested, we can estimate the contamination percentage assuming DHU is just below our LC-MS/MS detection limit. The calculation as performed using an estimated tRNA molecular weight of 28 kDa and approximately three DHU per tRNA[2].

References

1 Agilent Technologies, Inc, Agilent RNA Kits for the Agilent 2100 Bioanalyzer System, https://www.agilent.com/cs/library/datasheets/public/datasheet-rna-kits-bioanalyzer-5991-7891en-agilent.pdf.

2 P. Boccaletto, M. A. Machnicka, E. Purta, P. Piątkowski, B. Bagiński, T. K. Wirecki, V. de Crécy-Lagard, R. Ross, P. A. Limbach, A. Kotter, M. Helm and J. M. Bujnicki, MODOMICS: a database of RNA modification pathways. 2017 update, *Nucleic Acids Res*, 2018, **46**, D303–D307.