nature protocols

https://doi.org/10.1038/s41596-023-00857-0

Protocol

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A systems-level mass spectrometry-based technique for accurate and sensitive quantification of the RNA cap epitranscriptome

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Supplementary Information for:

CapQuant: A systems-level mass spectrometry-based technique for accurate and sensitive quantification of the RNA cap epitranscriptome

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Supplementary Methods

Enzymatic release, purification, and characterization of synthetic cap dinucleotides

● Timing **~3 d**

Procedure:

1. Digest the synthetic capped RNA oligo by incubating it in a 50 µl or 100 µl solution containing 2 units of nuclease P1, 30 mM sodium acetate pH 5.5, and 1 mM $ZnCl_2$ at 37 °C for 1 h.

2. Add equal volume of ice-cold chloroform:isoamyl alcohol 24:1 (v/v) to the above digestion mixture and vortex vigorously for 1 min. Centrifuge at 10,000*g* for 8 min at 4 °C and transfer the supernatant to a new 1.5-ml tube. Top up the supernatant to 100 µl by addition of ice-cold Milli-Q water.

3. Inject ~100 µl of the supernatant to a HPLC system and purify the resulting cap dinucleotides by ion-pairing HPLC on a 4.6 mm×250 mm Alltima HP C18 column (5 µm in particle size, Hichrom). Collect the fractions representing peaks eluting after 5'-AMP in 1.5-ml microcentrifuge tubes. See **Supplementary Figure 1** as an example.

4. Concentrate the individual fractions in a Speed-vac concentrator, which is operated with temperature uncontrolled, to dryness. Reconstitute in ~0.5 ml of ice-cold acetonitrile:water 3:7 (v/v) and concentrate again. Repeat the process three times. Reconstitute in 50 µl of Milli-Q water. Take out 5 µl, add 45 µl of Milli-Q water and mix well.

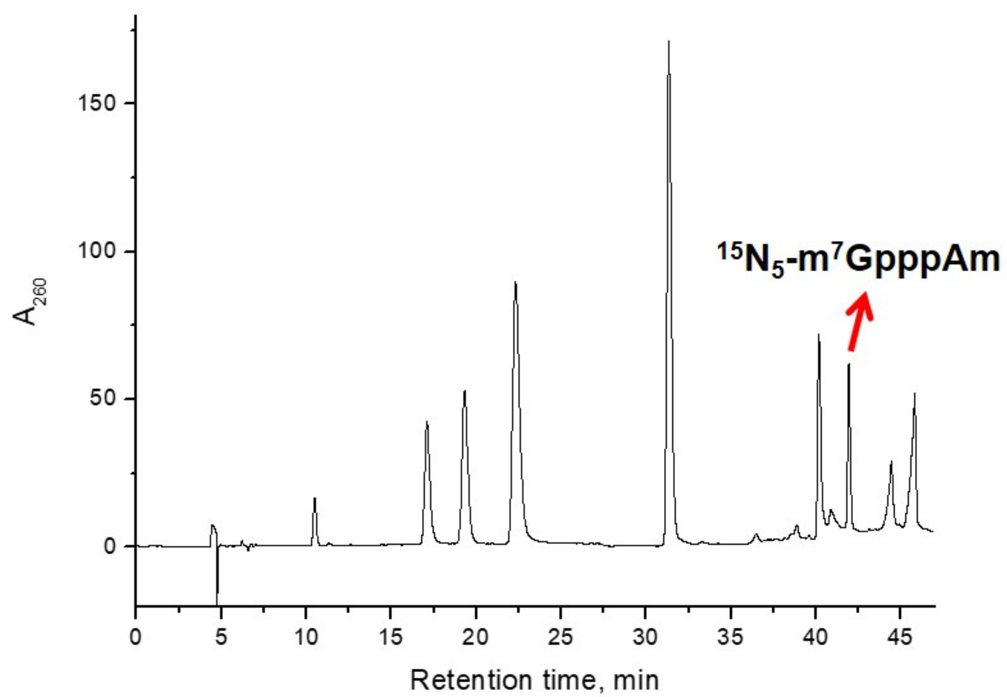
5. Inject 2-20 µl of the diluted fractions to a HPLC system coupled with a Triple quad mass spectrometer for analysis in multiple reaction monitoring (MRM) mode. The instrument conditions are configured as noted in the table below, which shows MRM transitions for $^{15}N_5$-m^{7}GpppAm. See **Supplementary Figure 2A** as an example.

LC part		**MS part**	
Inject volume	2-20 µl	Positive mode	MRM scan
Flow rate	0.2 mL/min	Gas temperature	350 °C
Column temperature	25°C	Gas flow	11 l/min
Mobile phase A	**8 mM NH_4HCO_3 pH 7.0**	Nebulizer	20 psi
Mobile phase B	**Methanol**	Sheath gas temperature	300 °C
Time/min	**Percentage of B/%**	Sheath gas flow	12 l/min
0	0	Capillary voltage	1.8 kV
5	0	Nozzle voltage	2 kV
15	40	Fragmentor voltage	135 V
15.1	0	ΔEMV	400 V
22	0	Collision energy	30 V
		MRM precursor ion ($^{15}N_5$-m^{7}GpppAm)	806
		MRM product ions ($^{15}N_5$-m^{7}GpppAm)	171, 136

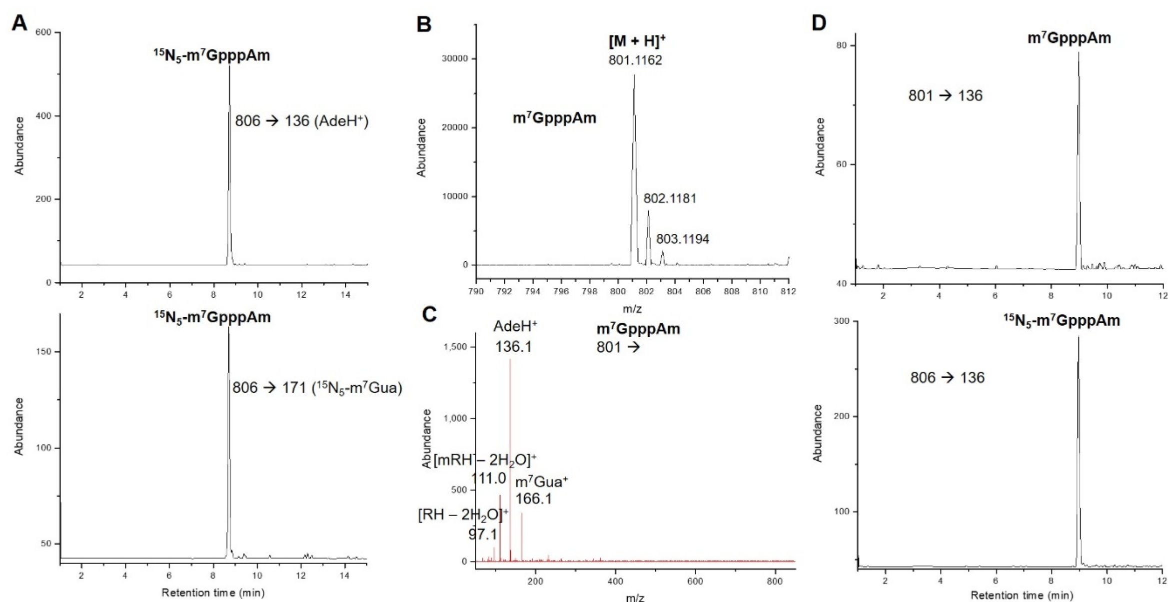
6. Determine the fragmentation pattern of the purified unlabeled cap dinucleotides that exhibited expected MRM transitions on a HPLC system coupled with a Triple quadrupole mass spectrometer in product ion scan mode. See the results in **Supplementary Figure 2C** and **Supplementary Figure 3**.

7. Confirm the identity of the purified unlabeled cap dinucleotides by high-resolution mass analysis on a Quadrupole time-of-flight mass spectrometer. See **Supplementary Figure 2B** as an example.

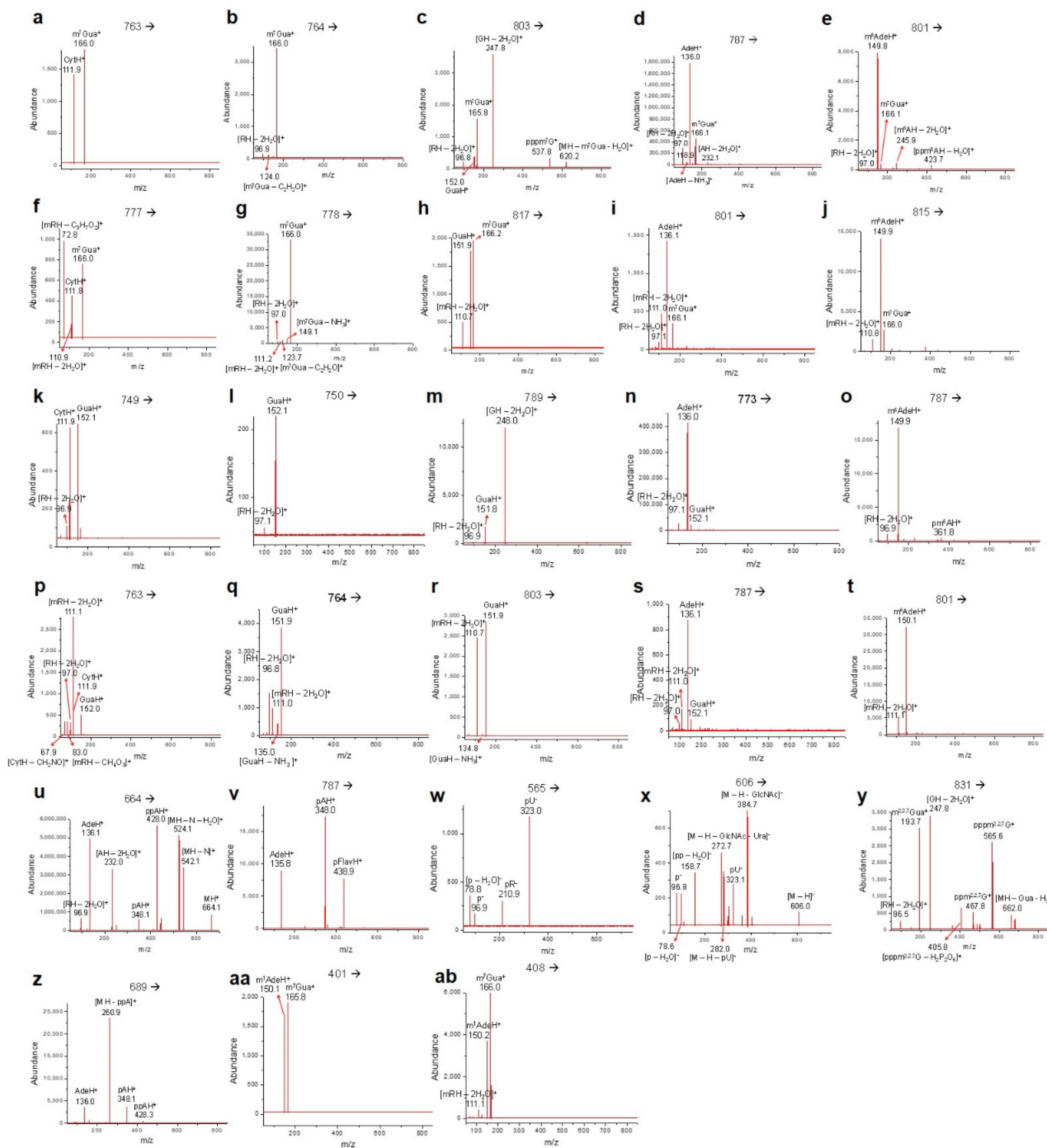
8. For each cap dinucleotide, prepare a mixture of the unlabeled version and $^{15}N_5$-labeled version. Analyze the mixture on a HPLC system coupled with a Triple quad mass spectrometer using instrumental parameters described in "Optimization of mass spectrometer parameters for targeted cap nucleotides" and 'Quantification of cap nucleotides by LC-MS/MS' sessions in the manuscript. See **Supplementary Figure 2D** as an example.



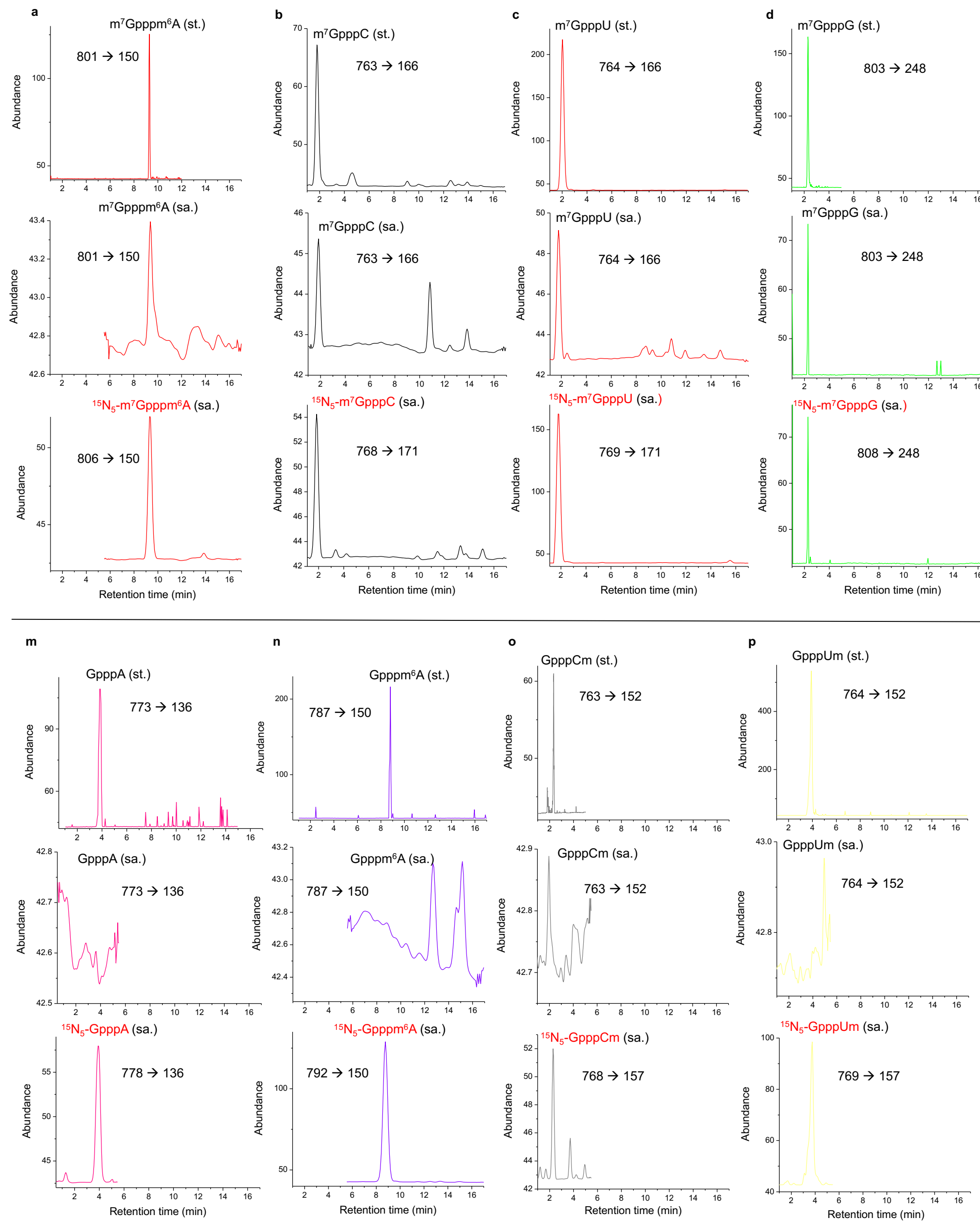
Supplementary Figure 1. HPLC purification of $^{15}N_5$-m^{7}GpppAm from the nuclease P1 digest of $^{15}N_5$-m^{7}GpppAm-capped RNA.



Supplementary Figure 2. Characterization of synthetic $^{15}N_5$-m^{7}GpppAm and m^{7}GpppAm by LC-MS analysis. (A) LC-MS/MS analysis of the HPLC fraction containing $^{15}N_5$-m^{7}GpppAm in MRM mode. **(B)** HRMS of m^{7}GpppAm. (**C**) LC-MS/MS analysis of m^{7}GpppAm in product ion scan mode. (**D**) LC-MS/MS analysis of a mixture of $^{15}N_5$-m^{7}GpppAm and m^{7}GpppAm in MRM mode. Ade, adenine; m^{7}Gua, *N*^{7}-methylguaninie; R, ribose; mR, 2-O-methylribose; H, hydrogen. The results in **C** have been published in the paper by Wang *et al.*[1]

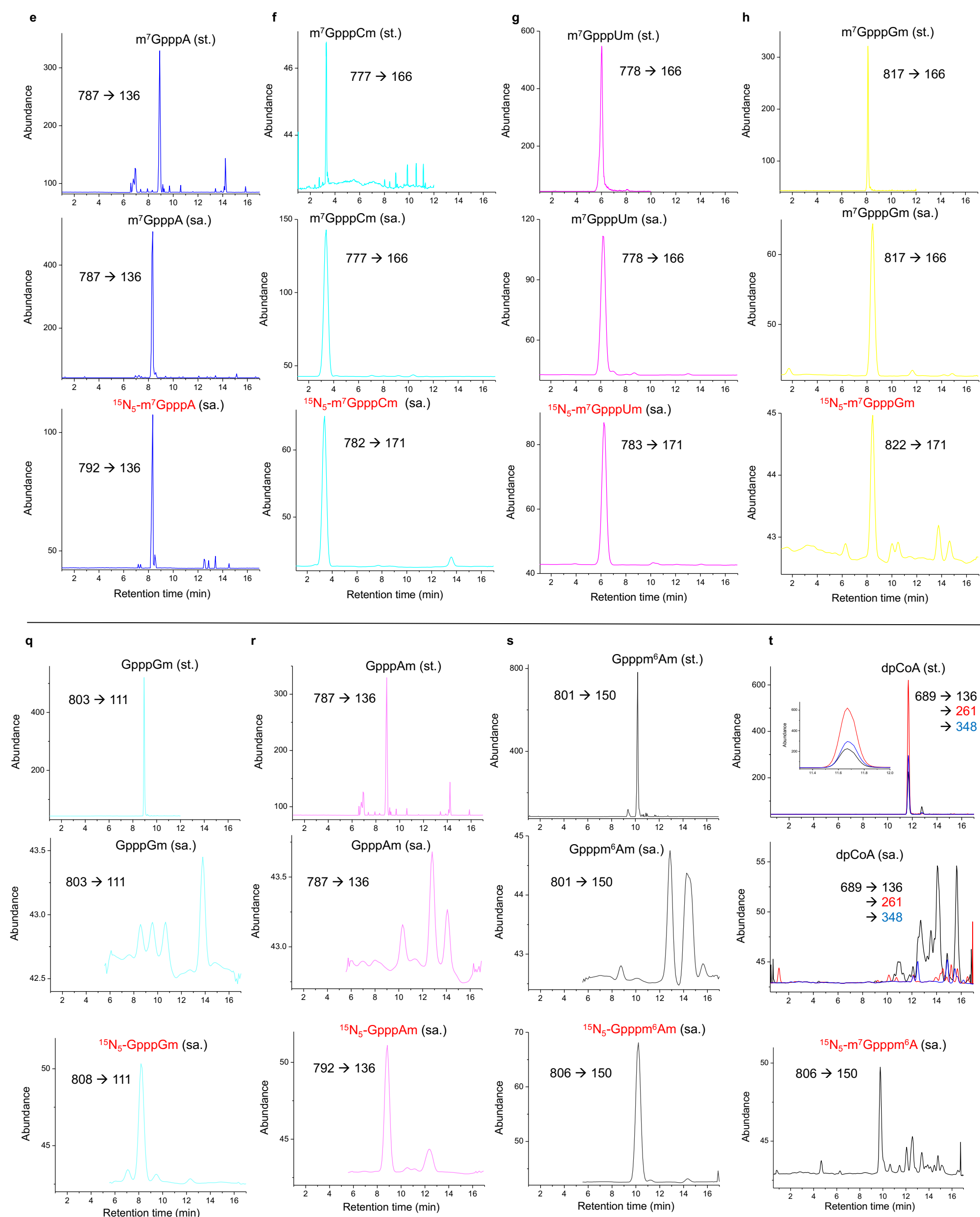


Supplementary Figure 3. MS/MS spectra of unlabeled cap nucleotides with assignments. (**A-AB**) m^{7}GpppC, m^{7}GpppU, m^{7}GpppG, m^{7}GpppA, m^{7}Gpppm^{6}A, m^{7}GpppCm, m^{7}GpppUm, m^{7}GpppGm, m^{7}GpppAm, m^{7}Gpppm^{6}Am, GpppC, GpppU, GpppG, GpppA, Gpppm^{6}A, GpppCm, GpppUm, GpppGm, GpppAm, Gpppm^{6}Am, NAD, FAD, UDP-Glc, UDP-GlcNAc, m^{2,2,7}GpppG, dpCoA, m^{7}Gpppm^{1}A, m^{7}Gpppm^{1}Am. Abbreviations: Ade, adenine; Gua, guanine; m^{7}Gua, N^7-methylguaninie; m^{6}Ade, N^6-methyladenine; m^{6}A, N^6-methyladenosine; Cyt, cytosine; G, guanosine; Flav, flavin; A, adenosine; N, nicotinamide; R, ribose; mR, 2-O-methylribose; H, hydrogen; p, phosphate; pp, diphosphate; ppp, triphosphate; N, nicotinamide; M, molecular ion; m^{1}Ade, N^1-methyladenosine; Ura, uracil; U, uridine; m^{2,2,7}Gua, $N^{2,2,7}$-trimethylguanine; m^{2,2,7}G, $N^{2,2,7}$-trimethylguanosine. The results have been published in the paper by Wang *et al*[1].

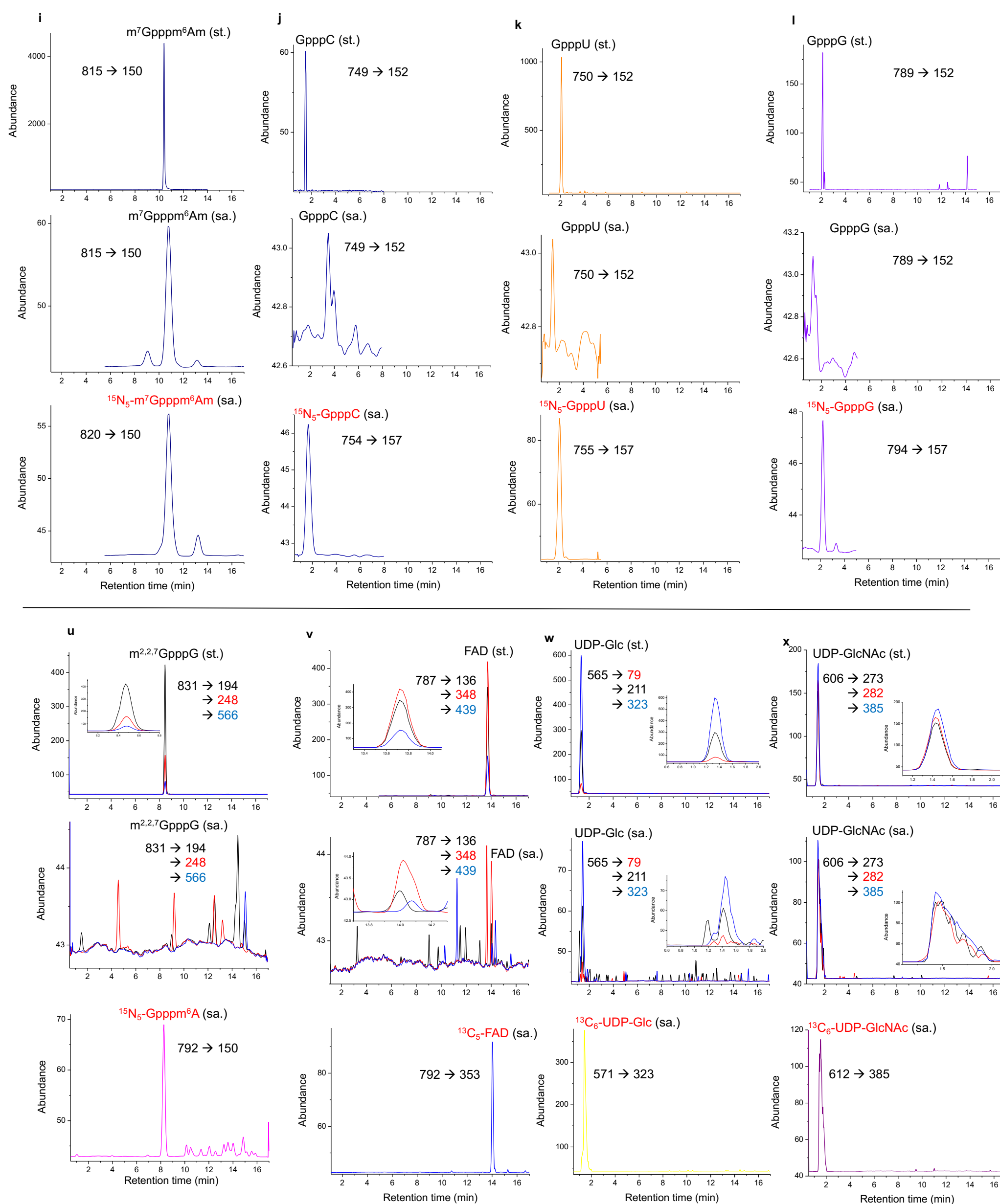


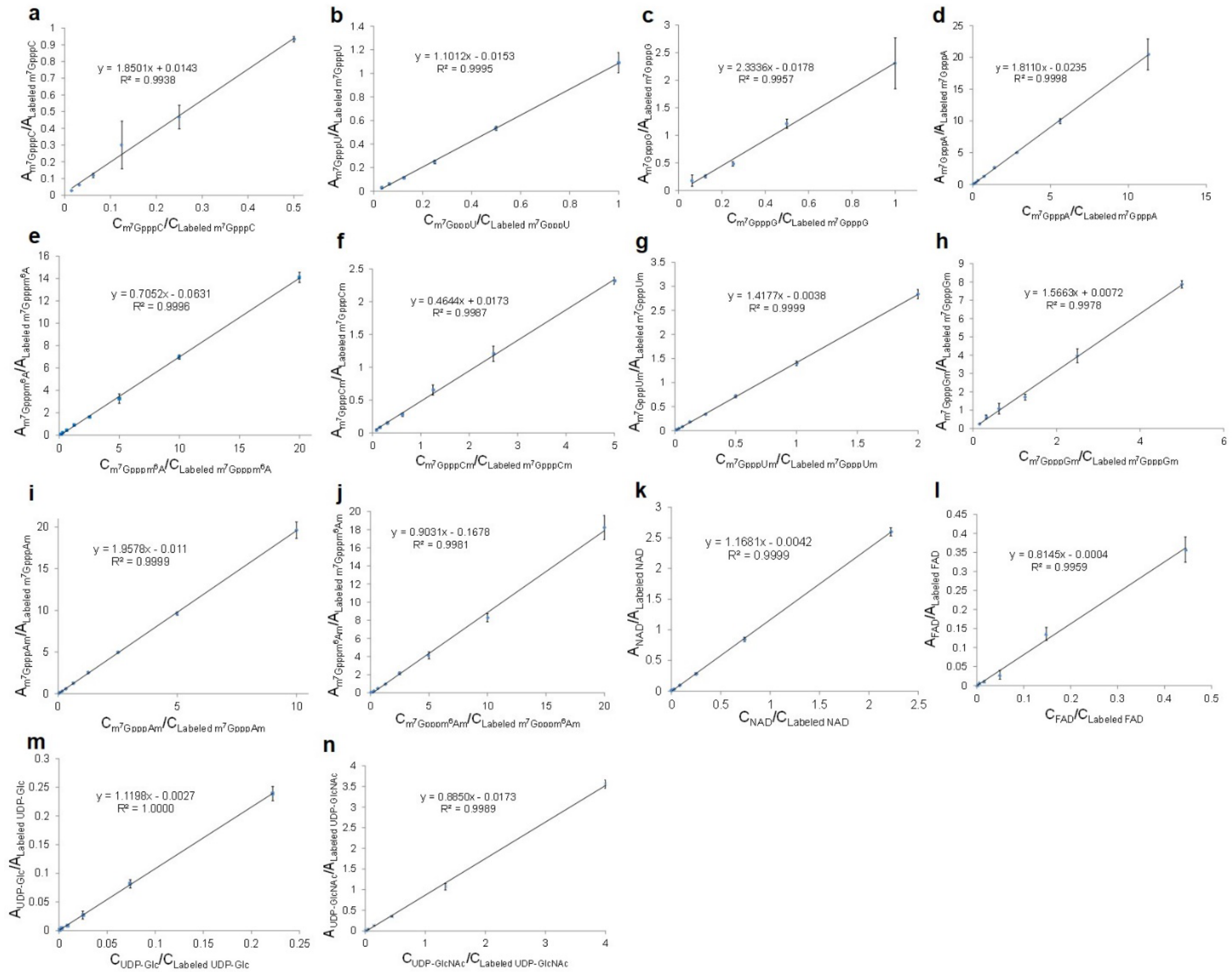
Supplementary Figure 4. Selective ion-chromatograms for monitoring MRM transitions for cap nucleotides. (**A-X**) m^7Gpppm^{6}A, m^7GpppC, m^7GpppU, m^7GpppG, m^7GpppA, m^7GpppCm, m^7GpppUm, m^7GpppGm, m^7Gpppm^{6}Am, GpppC*, GpppU*, GpppG*, GpppA*, Gpppm^{6}A*, GpppCm*, GpppUm*, GpppGm*, GpppAm*, Gpppm^{6}Am*, dpCoA*, $m^{2,2,7}$GpppG*, FAD, UDP-Glc, and UDP-GlcNAc. For each cap, there are three panels: top, unlabeled standard; middle, analyte in RNA sample; and bottom, isotope-labeled standard spiked into RNA sample. Red asterisks denote caps that were not detectable in any of the RNA samples analyzed in this work. The results have been published in the paper by Wang *et al.*[1]

Supplementary Figure 4, continued



Supplementary Figure 4, continued





Supplementary Figure 5. Calibration curves for the quantification of cap dinucleotides. (**A-N**) m^{7}GpppC, m^{7}GpppU, m^{7}GpppG, m^{7}GpppA, m^{7}Gpppm^{6}A, m^{7}GpppCm, m^{7}GpppUm, m^{7}GpppGm, m^{7}GpppAm, m^{7}Gpppm^{6}Am, NAD, FAD, UDP-Glc, UDP-GlcNAc. The results have been published in the paper by Wang *et al.*[1]

Reference

1. Wang, J. *et al.* Quantifying the RNA cap epitranscriptome reveals novel caps in cellular and viral RNA. *Nucleic Acids Res.* **47**, e130 (2019).