Supporting Information

for

"Simultaneous Quantification of Methylated Cytidine and Adenosine in Cellular and Tissue RNA by Nano-Flow Liquid Chromatography-Tandem Mass Spectrometry Coupled with the Stable Isotope-dilution Method"

By Lijuan Fu, et al. *Anal. Chem.*, 2015 (ac-2015-009515)

Table S1. Optimized instrument conditions for LC-ESI-MS^3 analysis of m^5C, Cm, m^6A and Am and detection limits for these modified nucleosides. The LOQ (limit of quantitation, defined as the amount of analyte that gives rise to a signal-to-noise ratio of 10) and the LOD (limit of detection, defined as the amount of analyte that gives rise to a signal-to-noise ratio of 3) values represent the means and standard deviations of the results from three measurements of the unlabeled standards. A constant activation time of 30 ms was employed for all the measurement.

Compounds	Transitions (MS^3 or MS^2)		Isolation Width	Optimized LTQ Parameters		LOD (amol)	LOQ (amol)
	Unlabeled	Isotope-labeled		Normalized Collision Energy	Activation Q		
C	244→112	247→115	3	37	0.25		
	112→ 95	115→ 97	2	40	0.50		
m^5C	258→126	263→126	2	37	0.25	2.8±0.4	9±2
	126→108	126→108	2	40	0.50		
Cm	258→112	263→112	2	37	0.25	2.2±0.7	7±2
	112→ 95	112→ 95	2	40	0.50		
A	268→136	273→136	3	35	0.25		
m^6A	282→150	285→153	3	39	0.31	0.7±0.2	1.9±0.6
	150→ 94	153→ 94	2	35	0.37		
Am	282→136	287→136	3	39	0.31	1.0±0.4	3.4±1.2
	136→ 94	136→ 94	2	35	0.37		

Table S2. Precision and accuracy for the measurements of m^5C, Cm, m^6A and Am from three validation runs. A 5-µL aliquot was injected in each run.

	intra-day		inter-day	
m^5C (nM)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)
0.125	8.7	99.8	6.4	99.4
1.25	12	89.6	7.7	92.4
6.25	3.0	102	0.3	102
Cm (nM)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)
0.113	4.8	94.4	4.6	96.1
1.136	8.0	90.4	5.7	94.2
5.68	4.6	99.7	0.9	99.0
m^6A (nM)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)
0.032	8.3	105	5.9	105
0.32	6.2	97.6	3.6	95.2
1.26	3.7	98.5	0.6	99.1
Am (nM)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)
0.025	2.9	100.2	4.8	102
0.25	4.2	96.9	2.0	99.2
1.03	4.7	104	7.0	96.6

Table S3. The stabilities of m^5C, Cm, m^6A and Am present in total RNA after three cycles of freeze (stored at -20°C for 24 hrs) and thaw (to room temperature). Shown are the percent recoveries and relative standard deviation (n = 3) obtained for the levels of the modified nucleosides measured after three cycles of freeze and thaw (relative to the levels of the modifications measured prior to freeze-and-thaw).

	total RNA	
	RSD (%)	Recovery (%)
m^5C	1.6	81.3
Cm	4.1	96.0
m^6A	4.1	122
Am	11	108

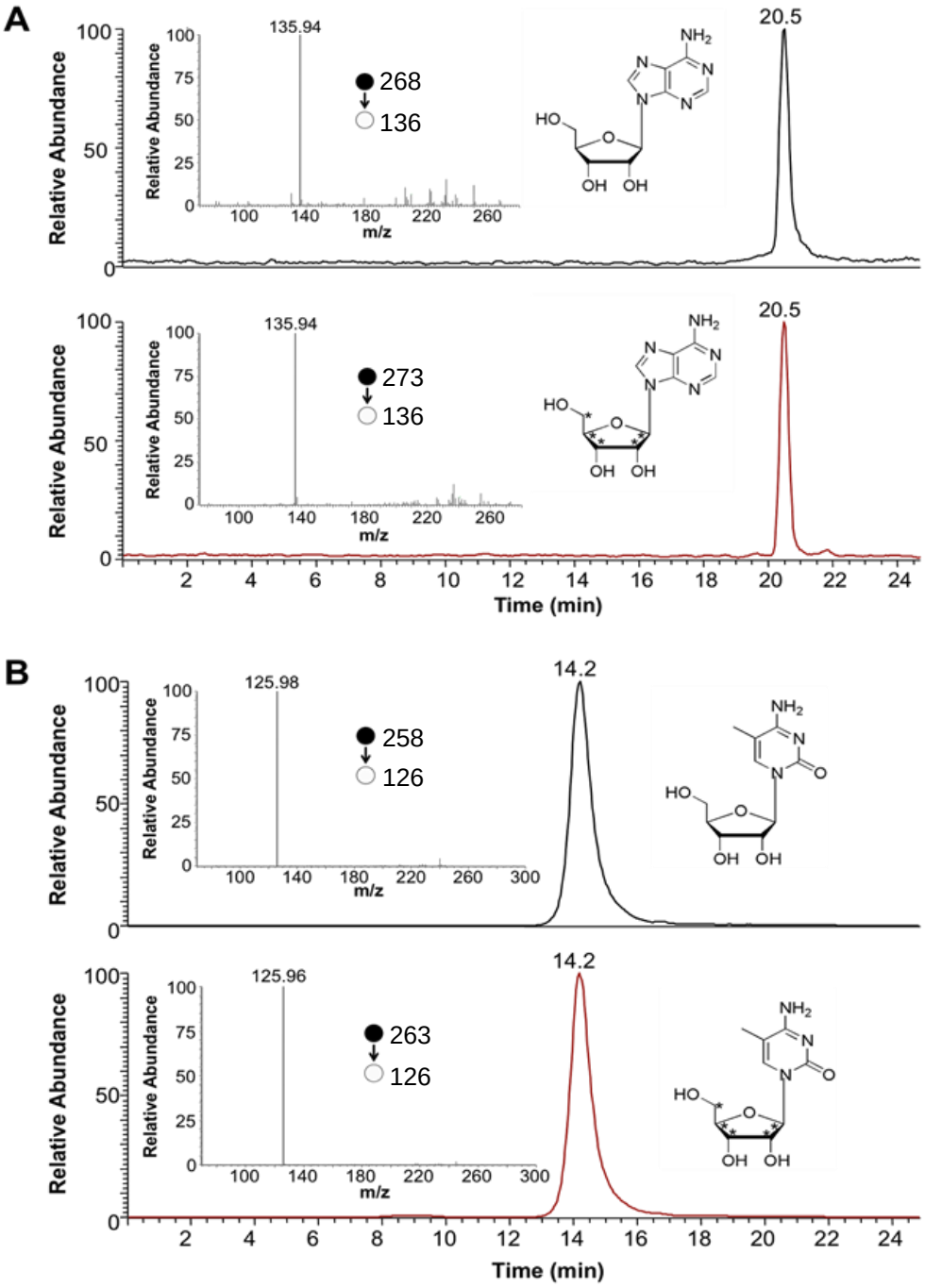

Figure S1. LC-MS/MS results for the analyses of unlabeled and purified stable isotope-labeled adenosine (A) and cytidine (B). Shown are the selective-ion chromatograms for monitoring the indicated transitions for the labeled and unlabeled nucleosides. The MS/MS are shown in the insert.

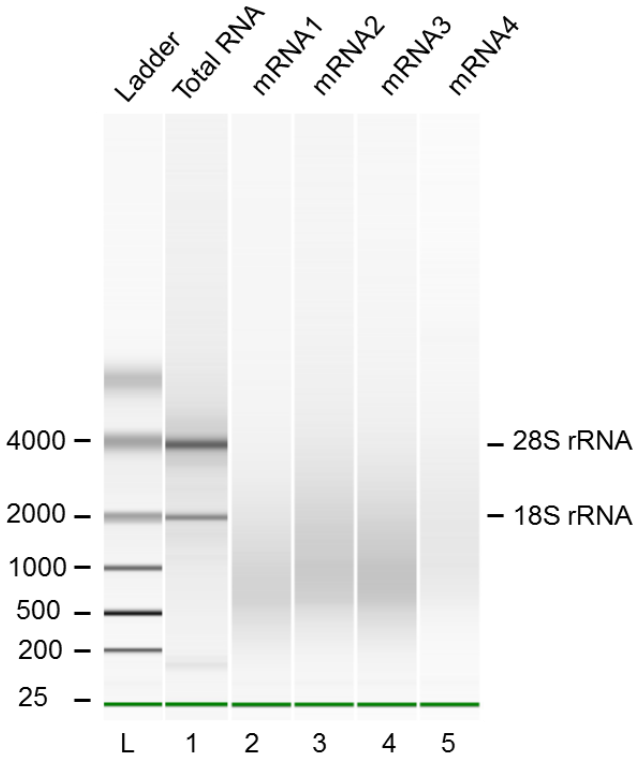

Figure S2. The quality of different batches of mRNA isolated from HEK293T cells, as determined by Agilent 2100 Bioanalyzer.

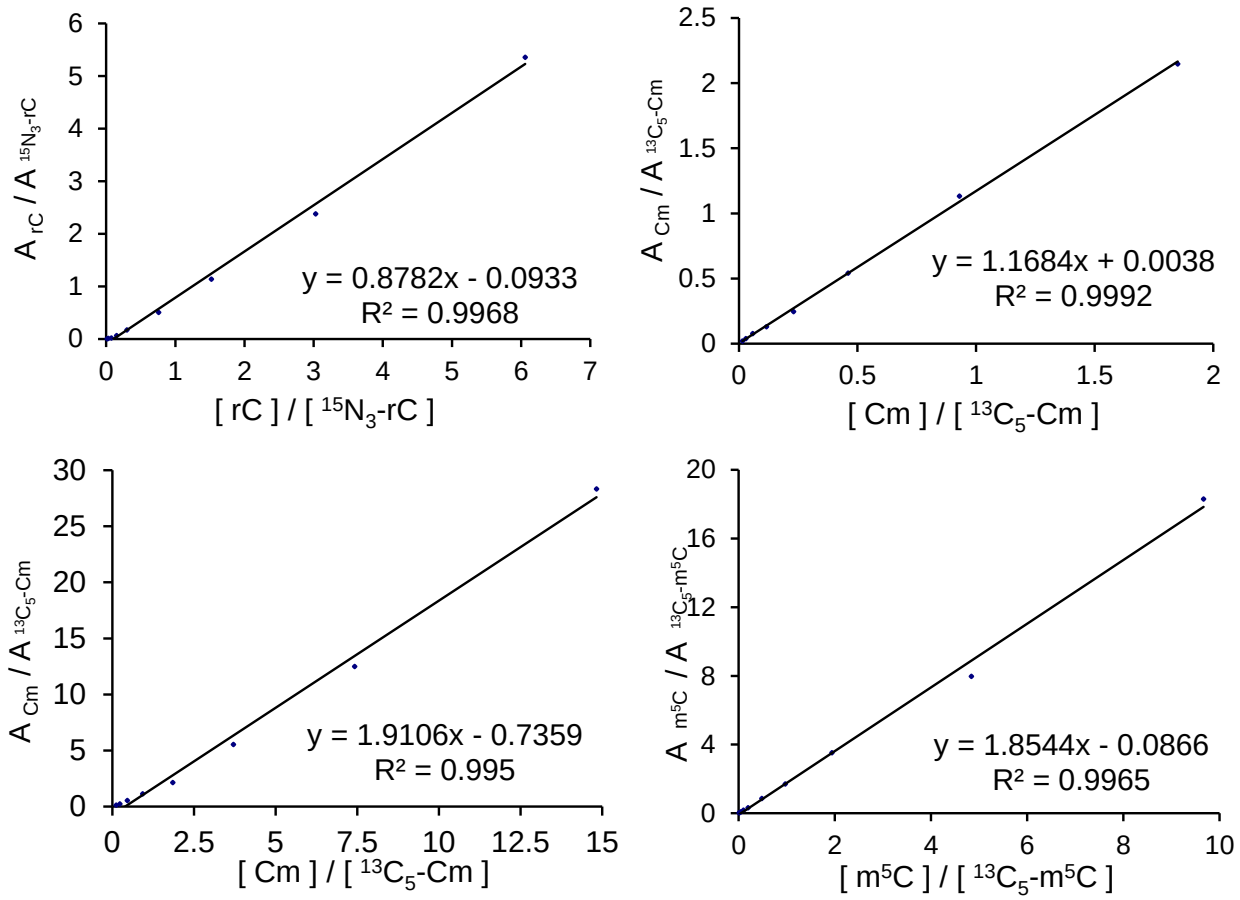

Figure S3. Calibration curves for the quantifications of rC, m^5C and Cm in RNA. The amounts of internal standards were 3300, 25.5 and 19.4 fmol, respectively, and the amounts of unlabeled rC, m^5C and Cm ranged 49.5 fmol - 20.0 pmol, 0.3 - 144.0 fmol and 0.2 - 246.6 fmol, respectively.

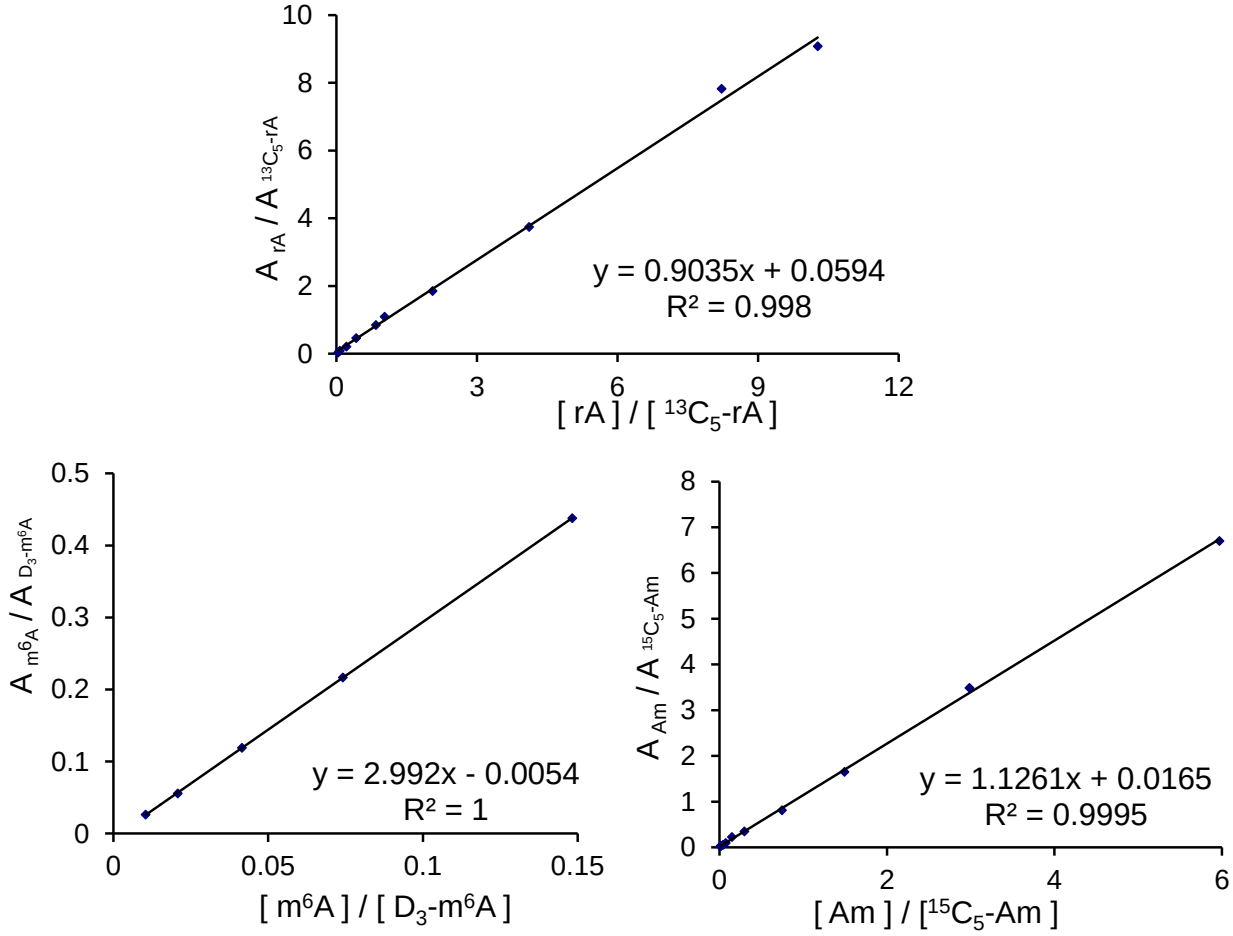

Figure S4. The calibration curves for the quantifications of rA, m^6A and Am in RNA. The amounts of internal standards were 1555, 8.5 and 6.9 fmol, respectively and the amounts of unlabeled rA, m^6A and Am ranged 52.9 fmol - 16.0 pmol, 0.09 - 1.2 fmol and 0.07 - 41.2 fmol, respectively.