Supporting Information

Quantitative analysis of ribosomal RNA modifications using stable isotope labeling and mass spectrometry

Anna M. Popova and James R. Williamson*

Department of Integrative Structural and Computational Biology and Department of Chemistry, The Scripps Research Institute, La Jolla, CA, USA

* Correspondence should be sent to James R. Williamson at jrwill@scripps.edu

Figure S1: Assignment of the isobaric rRNA fragments. Data were collected using positive or negative ion polarity. The ΔN value gives the number of ^{15}N-labeled nitrogen atoms. Bacterial strains lacking methyltransferase genes were obtained from the Keio single gene knockout library.

(A) LC-MS analysis of RNase A treated ^{14}N-70S ribosomes reveals two peaks with GCm nucleotide composition, eluting at different time points of the LC gradient and assigned to 23S: 1835-(m^2G)C-1836 and to16S: 527-(m^7G)C-528. To distinguish between the two possibilities, 70S sample was spiked with ^{15}N-23S RNA, enabling observation of ^{15}N isotopologues for all unique 23S fragments (red spectrum), leaving unique16S fragments 'unpaired' (blue spectrum). As a result, the peak at 22.2 min was assigned to 1835-(m^2G)C-1836 and the peak at 12.8 min to 527-(m^7G)C-528.

(B) We have observed an ^{14}N-^{15}N peak pair with GCmm nucleotide composition (red spectrum) referring to either 1401-G(m^4Cm)-1402 or to 966-(m^2G)(m^5C)-967 RNase A fragment from 16S RNA . Taking advantage of metabolic labeling with 5,6-D-uracil, the fragment was assigned to 966-(m^2G)(m^5C-967) (blue spectrum). Methylation at C5 of cytosine leads to the exchange of 5-D for a solvent proton which allowed us to distinguish between m^5C and m^4C residues using -1 Da mass shift (696 Da → 695 Da). 1401-G(m^4Cm)-1402 fragment with an expected mass shift shown by the green arrow (697 Da) was not observed experimentally. Most likely, due to ineffective RNase A cleavage at 2'-O-methylated residues (see also Table 2, Cm(2498) and Um(2552)).

(C-D) *E. coli* strains with genetically deleted RlmA and RlmB enzymes (blue spectra) were used to assign (C) an RNase A fragment at 682 Da to 23S: 745-(m^1G)Ψ-746 and (D) an RNase U2 fragment at 705 Da to 23S: 2251-(Gm)G-2252. ^{14}N- RNA isolated from the deletion strains was spiked with ^{15}N-labeled wild type 23S RNA, and the ^{15}N peaks lacking the ^{14}N isotopologue were used to confirm the identity of m^1G(745) and Gm(2251) nucleolytic fragments.

(E) Based on the MS data obtained using *ΔrlmKL* strain, two isobaric fragments 2444-G(m^2G)GGA(hU)-2449 and GGGGGU (^{14}N: 2051 Da, ^{15}N: 2078 Da for both) from RNase A digestion of 23S were assigned to the peaks eluting at 36.6 and 34.0 min correspondingly. Spectral overlaps observed in the ^{14}N peak region for 2444-G(m^2G)GGA(hU)-2449 preclude from reliable quantification of m^2G(2445) and hU(2449) modifications.

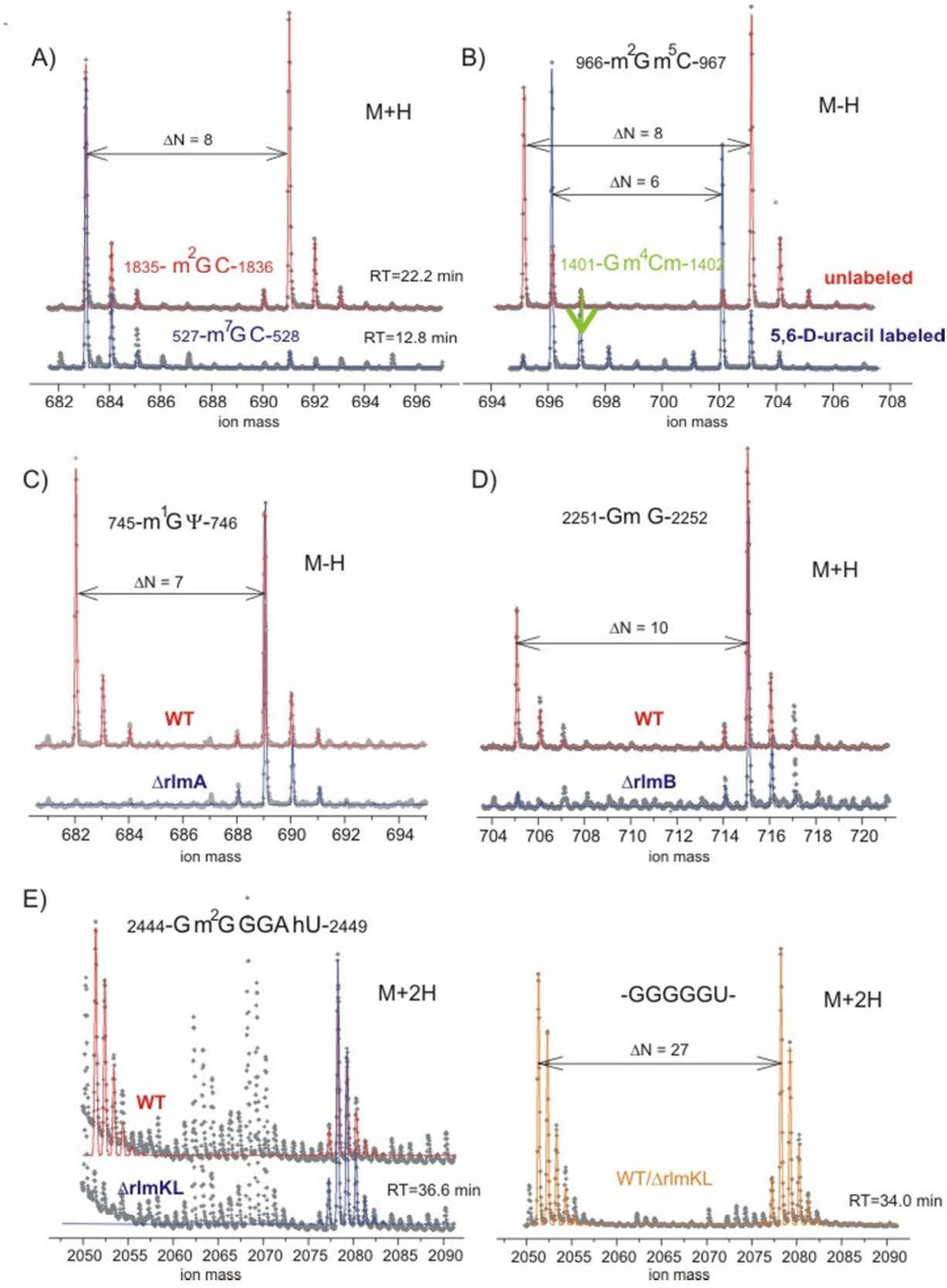
A)
M+H
ΔN = 8
1835- m2G C-1836
RT=22.2 min
527-m7G C-528
RT=12.8 min
682 684 686 688 690 692 694 696
ion mass
B)
966-m2G m5C-967
M-H
ΔN = 8
ΔN = 6
1401-G m4Cm-1402
unlabeled
5,6-D-uracil labeled
694 696 698 700 702 704 706 708
ion mass
C)
745-m1G Ψ-746
M-H
ΔN = 7
WT
ΔrlmA
682 684 686 688 690 692 694
ion mass
D)
2251-Gm G-2252
M+H
ΔN = 10
WT
ΔrlmB
704 706 708 710 712 714 716 718 720
ion mass
E)
2444-G m2G GGA hU-2449
M+2H
WT
ΔrlmKL
RT=36.6 min
2050 2055 2060 2065 2070 2075 2080 2085 2090
ion mass
-GGGGGU-
M+2H
ΔN = 27
WT/ΔrlmKL
RT=34.0 min
2050 2055 2060 2065 2070 2075 2080 2085 2090
ion mass

Table S1: Summary on identification of the modified rRNA fragments with non-unique composition.

Modification	RNase	Fragment identified	Alternative IDs	Method
16S RNA				
Ψ(516)	T1,U2	516-ΨG-517	23S: 955-ΨG-956	Fragments were monitored in the 30S fractions only. Pre-30S fractions exhibited over-stoichiometric amounts of these fragments (data not shown) suggesting presence of other Ψ containing RNAs.
	A	515-GΨ-516	23S: 1910-GΨ-1911	
m^7G(527)	A	527-(m^7G)C-528	23S: 1835-(m^2G)C-1836	Spiked with ^{15}N-labeled 16S/23S RNA, Figure S1 (A).
m^2G(966) m^5C(967)	T1	964-AU(m^2G)-966	23S: 2503-(m^2A)ΨG-2505	5,6-D-uracil labeled to distinguish U from Ψ. OR spiked with ^{15}N-labeled 16S/23S RNA.
	A	966-(m^2G)(m^5C)-967	16S: 1401-G(m^4Cm)-1402	5,6-D-uracil labeled to detect release of 5'-deuteron and observe C5-methylation. 1401-G(m^4Cm)-1402 wasn't detected, Figure S1 (B).
	U2	965-U(m^2G)-966	23S: 744-U(m^1G)-745 23S: 2068-U(m^7G)-2069	Spiked with ^{15}N-labeled 16S/23S RNA. IDs of the 23S fragments were not examined.
m^5C(1407)	U2	1406-U(m^5C)A-1408	23S: 1914-C($m^3Ψ$)A-1916	Spiked with ^{15}N-labeled 16S/23S RNA.
23S RNA				
m^1G(745) Ψ(746)	A	745-(m^1G)Ψ-746	16S: 1497-G(m^3U)-1498	5,6-D-uracil labeled to distinguish U from Ψ. 1497-G(m^3U)-1498 fragment wasn't detected. OR gene deletion of m^1G methyltransferase, Figure S1 (C).
m^2G(1835)	A	1835- (m^2G)C-1836	16S: 527-(m^7G)C-528	Spiked with ^{15}N-labeled 16S/23S RNA. See Figure S1 (A).
$m^3Ψ$(1915) Ψ(1917)	U2	1914-C($m^3Ψ$)A-1916	16S: 1406-U(m^5C)A-1408	Spiked with ^{15}N-labeled 16S/23S RNA.
Gm(2251)	U2	2251-(Gm)G-2252	23S: 2445-(m^2G)G-2446	Gene deletion of Gm methyltransferase, Figure S1 (D). 2445-(m^2G)G-2446 fragment wasn't detected.
m^2G(2445) hU(2449)	A	2444-G(m^2G)GGA(hU)-2449	23S: GGGGGU (774-779 or 855-860)	Gene deletion of m^2G methyltransferase, Figure S1 (E).
Cm(2498)	A	2497-A(Cm)C-2499	16S: 1407-(m^5C)AC-1409	5,6-D labeled to detect release of 5'-deuteron, and distinguish C5-CH_3 from 2'-OCH_3. 1407-m^5CAC-1409 fragment wasn't detected.
m^2A(2503) Ψ(2504)	T1	2503-(m^2A)ΨG-2505	16S: 964-AU(m^2G)-966	5,6-D-uracil labeled to distinguish U from Ψ. OR spiked with ^{15}N-labeled 16S/23S RNA.

Figure S2: Examples of MS data assigned to unique unmodified rRNA fragments. ^{14}N - ^{15}N peak pairs assigned to compositionally unique unmodified 23S RNA fragments from the T1 digestion of 70S. Peak amplitudes were used to compute RNA level values (f) for each fragment, which then were averaged to obtain the total RNA level (f_{total} = <f>= 0.34). f_{total} was used as normalization for the f values of modified fragments (f_{mod}): f_{mod} /f_{total}. MS data were collected in the positive mode.

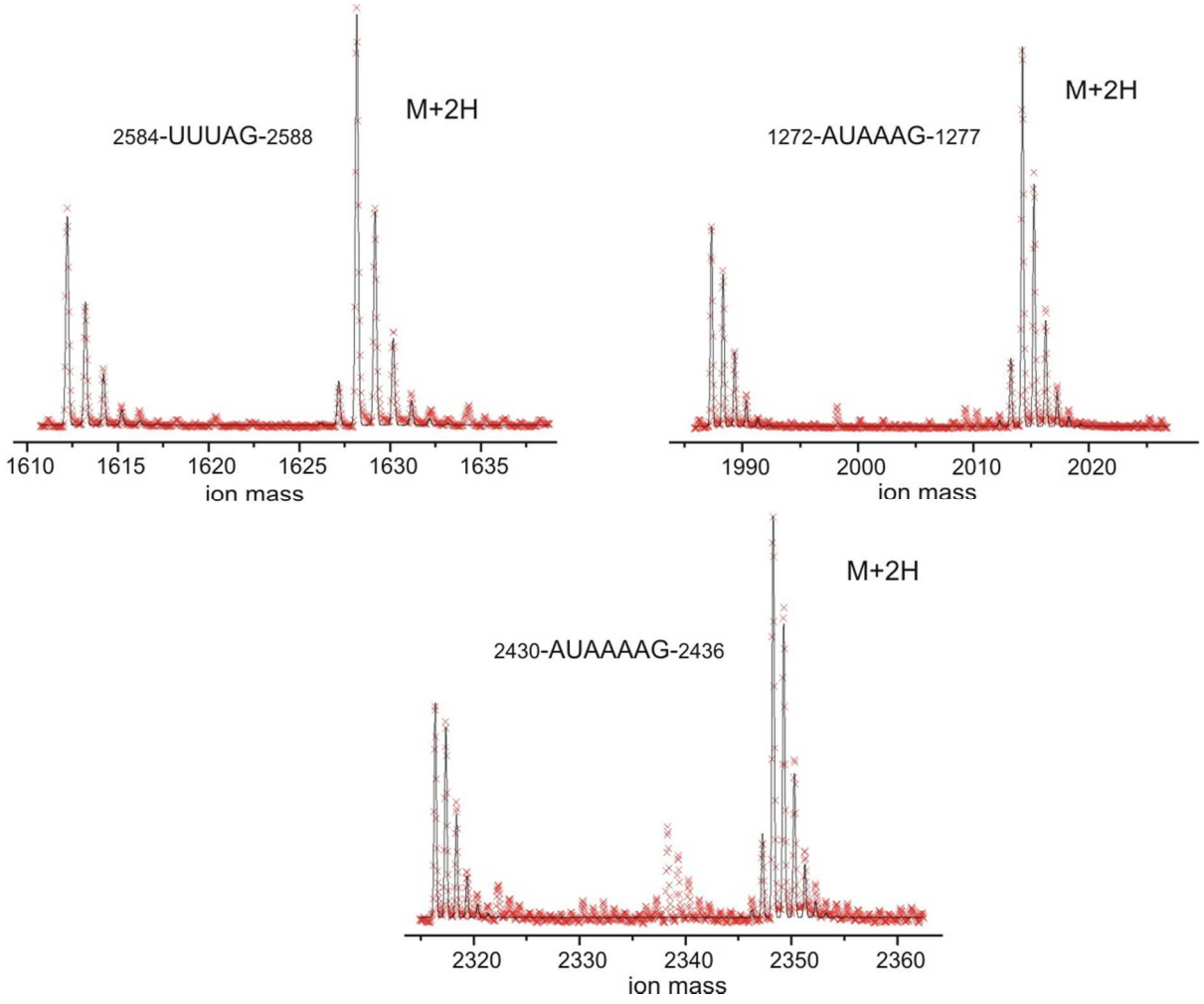

Table S2: Ribosomal RNA modifications that have not been identified in this work. The nucleolytic fragments predicted from a theoretical rRNA digestion are shown. The fragments are likely either too short (e.g., 2457-Ψ-2457) or, possibly, co-elute with other digestion products, which precludes from their MS identification.

Modification	RNase	Sequence
23S RNA		
Ψ (955)	T1, U2 A	955-ΨG -956 952-GGGΨ-955
Ψ (2457)	T1, U2 A	2456- CΨG-2458 2457- Ψ-2457
Ψ (2580)	T1, U2 A	2579- CΨG-2581 2580- Ψ-2580
Ψ(2604) Ψ(2605)	T1, U2 A	2604-ΨΨCG-2607 2602-AGΨ-2604 2605- Ψ-2605

Figure S3: Cluster analysis of 16S RNA modification profiles from three replicate experiments. In order to demonstrate the reproducibility of the quantitation, the relative amounts of modified 16S RNA fragments in the sucrose gradient fractions collected across the 30S peak from three independent experiments are shown. Cluster analysis reveals three groups of 16S RNA modifications.

Upon closer consideration, m^4Cm(1402) residue may form an independent group as its inventory profiles vary between the experiments. When data for each experiment are analyzed separately (not shown), residue 1402 co-clusters with group II (Experiment 1), group III (Experiment 2, data plotted in Figure 4 of the main text), or form a separate group (Experiment 3).

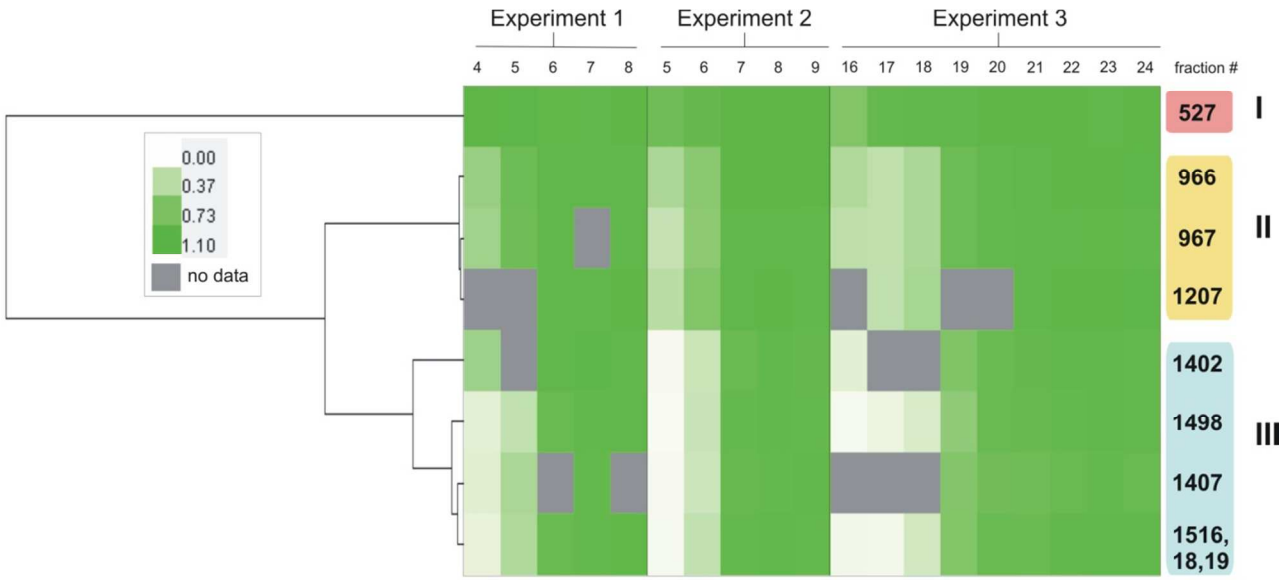

Figure S4: Cluster analysis of 23S RNA modification profiles from four replicate inventory experiments. Normalized RNA modification levels are shown across the 50S and 70S peaks obtained using four replicate sucrose gradients. The analysis reveals two groups of 23S modifications (red and cyan). Figure 5 of the main text represents RNA modification levels from Experiment 4.

A more detailed consideration of the data may suggest the presence of four clusters of RNA modifications: IA - [1618, 2030, 1962, 1835, 745-747, 2503-04, 2251, 2069], IB - [1939, 2498-2501], IIA - [2552] and IIB - [1911-17]. Modifications forming cluster IA are present at the highest level in the pre-50S/70S fractions, cluster IB modifications exhibit slightly lower levers, and modifications in clusters IIA and IIB are depleted. Accordingly, cluster IA residues are modified earlier on the assembly pathway than cluster IB residues, followed by modifications at 2552 and 1911, 15, 17 that occur at the late stages of ribosome assembly. Further studies might be useful to reveal whether cluster I modifications (red) can be separated into two individual clusters (IA and IB) and to better understand the timing of these modifications during the 50S subunit assembly.