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The Activity of rRNA Resistance Methyltransferases Assessed by MALDI Mass Spectrometry

Stephen Douthwaite, Rikke Lind Jensen, and Finn Kirpekar

Summary

Resistance to antibiotics that target the bacterial ribosome is often conferred by methylation at specific nucleotides in the rRNA. The nucleotides that become methylated are invariably key sites of antibiotic interaction. The addition of methyl groups to each of these nucleotides is catalyzed by a specific methyltransferase enzyme. The Erm methyltransferases are a clinically prevalent group of enzymes that confer resistance to the therapeutically important macrolide, lincosamide, and streptogramin B (MLS_B) antibiotics. The target for Erm methyltransferases is at nucleotide A2058 in 23S rRNA, and methylation occurs before the rRNA has been assembled into 50S ribosomal particles. Erm methyltransferases occur in a phylogenetically wide range of bacteria and differ in whether they add one or two methyl groups to the A2058 target. The dimethylated rRNA confers a more extensive MLS_B resistance phenotype. We describe here a method using matrix-assisted laser desorption/ionization (MALDI) mass spectrometry to determine the location and number of methyl groups added at any site in the rRNA. The method is particularly suited to studying *in vitro* methylation of RNA transcripts by resistance methyltransferases such as Erm.

Key Words: rRNA methylation; ribosomal antibiotic resistance; RNA mass spectrometry.

1. Introduction

Many clinically important antibiotics inhibit the growth of bacteria by blocking protein synthesis on the ribosomes ***(1–3)***. These antibiotics bind to regions of the ribosome that are concerned with essential steps in protein synthesis such as peptide bond formation, GTP hydrolysis, and mRNA decoding. The main contact sites for the antibiotics are on the rRNA, rather than on the ribosomal protein components ***(4)***, which is consistent with the

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view that the rRNA carries out the primary functions of the ribosome, including formation of the peptide bond ***(5,6)***. Not surprisingly therefore, changes in the ribosome structure that confer antibiotic resistance are mainly to be found in the rRNA and consist of nucleotide methylations or base substitutions ***(4)***. There are indeed cases of ribosomal protein (r-protein) mutations that confer resistance to ribosome-targeting antibiotics. However, these mutations tend to confer resistance in an indirect manner by influencing the conformation of adjacent rRNA structures that make contact with the antibiotic ***(7,8)***.

In pathogenic bacteria with multiple rRNA (*rrn*) operons, resistance to ribosome-targeting drugs is most commonly conferred through modification of the rRNA by specific methyltransferase enzymes ***(9,10)***. All the rRNA resistance methyltransferases studied to date use S-adenosyl-L-methionine (AdoMet) as the methyl group donor and contain conserved motifs involved in AdoMet binding ***(11)*** and have distinct similarity to Rossmann-fold structures found in proteins that bind other adenosine-based cofactors such as ATP and NAD ***(12)***. In other parts of their structures, the rRNA methyltransferases are largely heterogeneous, and these differences presumably enable the enzymes to distinguish their specific target nucleotides.

Target nucleotides in 16S rRNA tend to be methylated after assembly of the 30S subunit. The methyltransferases Grm and KamA function in this manner by methylating the assembled 30S subunit at nucleotides G1405 and A1408, respectively, and thereby confer resistance to aminoglycoside antibiotics ***(13,14)*** (*Escherichia coli* rRNA nucleotide numbering is used throughout). These target nucleotides are displayed at the decoding region on the subunit interface sites on the mature 30S subunit ***(15,16)***. For methylation to occur, the nucleotides are not only required to be accessible on the surface of the small subunit, but also need to be presented in higher-order structures that are absent in the free 16S rRNA. In contrast, the free 23S rRNA is generally the preferred substrate for methylation prior to its complete assembly with r-proteins to form 50S subunits. Examples include nucleotide G748, the target for the tylosin resistance methyltransferase RlmA^{II} (TlrB) ***(17)***; nucleotide A1067, the target for the thiostrepton resistance methyltransferase Tsr ***(18,19)***; nucleotide A2058, the target for the MLS_B (macrolide, lincosamide, and streptogramin B antibiotic) resistance methyltransferase Erm ***(20,21)***; and nucleotides G2470, U2479, and G2535 that are respectively targeted by the orthosomycin resistance methyltransferases EmtA ***(22)***, AviRb, and AviRa ***(23)***. In addition to methylating the free 23S rRNA substrate, these methyltransferases also specifically recognize their targets within short RNA transcripts, making them ideal for the type of *in vitro* studies described here.

The most pervasive of the resistance methyltransferases are those belonging to the Erm family. All Erm methyltransferases specifically methylate the N^6-position of nucleotide A2058 in 23S rRNA, but differ as to whether they monomethylate or dimethylate this nucleotide ***(20,24)***. Erm monomethyltransferases are found predominantly in drug-producing actinomycetes species and confer the MLS_B type I phenotype with high resistance to lincosamides, low to moderate resistance to macrolide and streptogramin B antibiotics ***(24,25)***, but no resistance to the latest generation of macrolides, the ketolides ***(26)***. Erm dimethyltransferases confer the MLS_B type II phenotype with high resistance to all macrolide, lincosamide, and streptogramin B antibiotics ***(24,27)*** including ketolides ***(26)***. Type II MLS_B resistance with dimethylation of the rRNA is the more common mechanism in bacterial pathogens.

Nucleotide A2058 is situated in the peptidyl transferase loop of 23S rRNA. This region is inaccessible to Erm methyltransferases in assembled 50S subunit particles, although precursor particles can serve as substrates for ErmC ***(28)***. Nucleotide A2058 is methylated by Erm(E) ***(29)*** and Erm(S) ***(30)*** when displayed in small *in vitro* RNA transcripts of 26 to 48 nucleotides. However, methylation is much more efficient in slightly larger RNA transcripts that maintain the structure of the peptidyl transferase loop (Fig. 1); such substrates are methylated as efficiently as the intact 23S rRNA and are thus well-suited for studying Erm methyltransferase activity. Methylation activity can be determined by a number of techniques including the use of radiolabeled AdoMet ***(30)***, primer extension with reverse transcriptase ***(29)***, and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) ***(17,23)***. Each method has its own strengths and weaknesses, and one of the advantages with the MALDI-MS approach is the ability to distinguish between mono- and dimethylation at the Erm target nucleotide A2058. Here we describe the preparation of the dimethyltransferase Erm(E) for *in vitro* activity studies on RNA transcripts (Fig. 1) followed by MALDI-MS analysis of methylation. Detection of singly methylated A2058 by MALDI-MS is demonstrated using the Erm(N) monomethyltransferase (formerly TlrD) ***(27)***.

2. Materials

1. Lauria Bertani (LB) broth ***(32)*** was used as the rich medium for bacterial cultures. Single-distilled water was used for media, as well as for gels and running buffers; double-distilled water was used for all other buffers and solutions.
2. TMN: 50 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 100 mM NH_4Cl.
3. Buffer A: 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 M NH_4Cl, 10% glycerol, 6 mM β-mercaptoethanol. Buffer B: 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$,

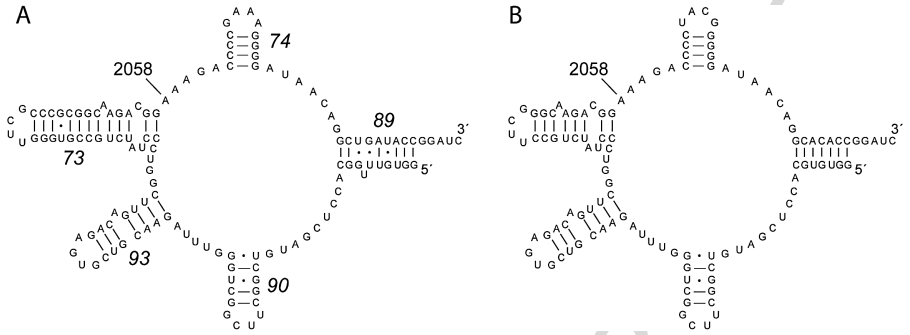


Fig. 1. *In vitro* RNA transcripts representing the peptidyl transferase loop of 23S rRNA. The structures are based on the *E. coli* 23S rRNA sequence, although the majority of nucleotide positions are highly conserved and thus identical in most bacteria ***(43)***. Helices 73, 74, 89, and 90 have been shortened in the *in vitro* transcripts, and the missing sequences have been replaced with stable tetraloops (except for helix 89). The 3′- and 5′-ends of the structures are positioned in helix 89; this ensures that helix 73 will be stably formed and maintain the structure at the target nucleotide A2058. In structure B, helices 73 and 89 have been shortened further, and the tetraloop capping helix 74 has been altered to give a unique RNase T1 fragment containing the A2058 target (AAAG). Testing with Erm(E) and Erm(N) shows that RNA structures such as these, which contain the complete peptidyl transferase loop and most of helix 73, are methylated as efficiently as intact 23S rRNA.

6 mM β-mercaptoethanol. Buffer C: 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 100 mM NH_4Cl, 10% glycerol, 6 mM β-mercaptoethanol.

4. Sodium dodecyl sulphate (SDS) gels were used to check the size and purity of the Erm methyltransferase. The stacking gel was 4% and the separation gel 12% polyacrylamide (19:1 acrylamide: *bis*-acrylamide, electrophoresis grade); protein bands were stained with Coumassie Blue.
5. T7 RNA polymerase (Promega), RNAguard (Amersham), and enzymes for DNA manipulations (New England Biolabs) were used according to the suppliers' recommendations. Buffers were obtained from the enzyme suppliers.
6. TSC buffer (5X concentrated): 200 mM Tris-HCl, pH 7.9, 30 mM $MgCl_2$, 50 mM dithiothreitol, 20 mM spermidine.
7. NTP mix: 2.5 mM each of ATP, GTP, UTP, and CTP, pH 7.5.
8. Phenol was distilled and equilibrated with water; stored at –20 °C.
9. Gels for checking RNA transcripts were 10% polyacrylamide (19:1 acrylamide: *bis*-acrylamide, electrophoresis grade) containing 90 mM Tris-borate, pH 8.3, and 1 mM EDTA. Samples were loaded with 1/5 volume of 10% Ficoll 400 containing 0.05% xylene cyanol and 0.05% bromophenol blue marker dyes. After

electrophoresis, gels were stained by soaking for 1 h in 5% acetic acid containing 0.1% toluidine blue, and then destained in 5% acetic acid.

10. RNase A was from Sigma-Aldrich, and RNase T1 was from US Biochemicals.
11. 3-Hydroxypicolinic acid (3-HPA; from Sigma-Aldrich) was dissolved in a 1:1 mixture of HPLC-grade acetonitrile and HPLC-grade water to a concentration of 0.5 M. As well as being used for MALDI matrix preparation, the 3-HPA solution also serves as an excellent RNA denaturing agent for the RNase digestion steps.
12. Poros 50R3 chromatography resin (Applied Biosystems) was suspended in HPLC-grade methanol to approximately 0.2 g/mL.
13. Triethylammonium acetate solution (used at 1 M concentration, pH 7.0) was from Merck.
14. Dowex AG 50W-X8 cation exchange material (acid form) was from BioRad. The material was converted to its ammonium form by five repeated incubations with two volumes of 10 M ammonium acetate, followed by five repeats of washing with two volumes of HPLC-grade water. The cation exchange material was resuspended in approximately two volumes of HPLC-grade water.

3. Methods

3.1. Preparation of Erm(E) Methyltransferase

1. Add 0.5 mL of an overnight culture of *E. coli* cells harboring a plasmid such as pJEK47 encoding *erm(E)* (see Notes 1 and 2) to 200 mL of LB broth, containing 100 µg/mL of ampicillin, in a 1 L flask. Shake at 100 rpm in incubator at 37 °C, and measure the optical density (A_{450}) every 30 min. Draw a semi-log curve to follow the cell growth. Place the cells on ice when they reach an A_{450} of 0.4. All the buffers, tubes, and centrifuge rotors should be between 0 °C and 4 °C for the rest of this section.
2. Harvest the cells by centrifugation at 10,000X g for 10 min in a Beckman JA14 rotor, or equivalent. Carefully pour off the supernatant. Wash the cells by resuspending in 200 mL of cold TMN buffer and repeating the centrifugation step. Pour off the supernatant, and resuspend the cells in 20 mL of TMN buffer. Transfer the cell suspension to suitably sized polyethylene tubes (JA20 tubes) on ice.
3. The cell walls are lyzed by sonication, keeping the tube on ice. Wear gloves and ear protection; rinse the sonicator probe with ethanol, and dry before and after use. Sonicate with four bursts at approximately 150 W for 30 s (with a 30-s pause between each burst, or longer if the probe begins to heat up).
4. The cell debris is removed by centrifuging at 30,000X g for 10 min. Transfer the supernatant, containing ribosomes and the Erm(E) methyltransferase, to fresh, cold JA20 tubes. Centrifuge again, to remove any remains of cell walls and cell membranes, and transfer the supernatant to cold Ti50 ultracentrifuge tubes. Fill and balance tubes with cold TMN buffer, and centrifuge at 100,000X g for 3 h at 4 °C in an ultracentrifuge. This, and the following, ultracentrifugation step can be carried out at 20,000X g overnight if the timing is more convenient (see Note 3).

5. Pour off the supernatant. Keep the tubes on ice while redissolving the ribosome pellets by gentle pipetting in 2.5 mL of buffer A. Allow to stand on ice between 2 and 5 h to wash off the methyltransferase.
6. Centrifuge at 100,000X g for 3 h at 4 °C in Ti50 ultracentrifuge tubes. The ribosomes will pellet leaving the methyltransferase in the supernatant. Collect the supernatant and transfer to a dialysis tube. Seal the tube, excluding air. Dialyze against 200 mL of buffer B in a cold room (4 °C) with stirring; change the buffer every hour (four times in all).
7. Transfer the dialyzed supernatant to Eppendorf tubes. Pellet the methyltransferase by spinning at full speed in an Eppendorf centrifuge (15, 0000 to 20, 000 rpm) for 20 min at 4 °C. Remove the supernatant, keep 15 μL for SDS gel analysis, and discard the rest. Gently redissolve each pellet in 50 μL of buffer C (see Note 3). Take out a total of 15 μL for SDS gel analysis for size (see Note 2) and purity (see Note 4). The rest of the methyltransferase can be stored at –20 °C.

3.2. Transcription of RNA Methylation Substrate

1. The DNA templates for transcription of the RNA methylation substrates (Fig. 1) have been assembled from oligodeoxynucleotides, which have then been cloned into a pGEM plasmid after a T7 RNA polymerase promoter. A restriction site sequence (in this case *Bam*HI) has been incorporated at the 3´-end of the template sequence to facilitate run-off transcription.
2. Double-stranded plasmid DNA containing one of these sequences (Fig. 1) is prepared by standard methods. To 10 μg plasmid DNA in an Eppendorf tube add: 10 μL of 10X *Bam* HI digestion buffer; H_2O to bring the total volume to 100 μL; and 10 U of *Bam*HI enzyme. Leave overnight at 37 °C in a warm air incubator.
3. Add 200 μL of 0.25 M sodium acetate followed by 750 μL 96% ethanol. Leave in a –20 °C freezer for 1 h. Spin at full speed in an Eppendorf centrifuge (15, 0000 to 20, 0000 rpm) for 20 min at 4 °C. Remove supernatant and wash pellet with 100 μL 70% ethanol. Remove supernatant, dry pellet, and redissolve in 50 μL of H_2O (see Note 5). Check 1 μL of the restricted DNA on a 1% agarose gel alongside 0.1 to 0.2 μg of the uncut DNA.
4. For the T7 transcription reaction, add the following to an Eppendorf tube: 20 μL of 5X TSC buffer; 25 μL of NTP mix; 49 μL of *Bam*HI-cut DNA; 50 U (2.5 μL) of T7 RNA polymerase; and 100 U (3.2 μL) of RNA guard. Incubate at 37 °C for at least 2 h (see Note 6). Stop the transcription by adding 200 μL of H_2O and 300 μL of phenol; vortex 30 s. Recover the RNA by centrifuging the phenol mixture for 1 min and taking the aqueous (upper) phase to a fresh tube. Repeat the extraction procedure with 300 μL of phenol/chloroform (1:1, vol:vol) and then with 300 μL of chloroform.
5. Add 0.1 volume of 2.5 M sodium acetate followed by 2.5 volumes of ethanol to the recovered aqueous phase. Leave in the –20 °C freezer for at least 1 h. Spin at full speed in an Eppendorf centrifuge (15, 0000 to 20, 0000 rpm) for 20 min

at 4 °C. Remove supernatant and wash the pellet with 100 µL of 70% ethanol. Remove supernatant, dry the pellet, and redissolve in 50 µL of H_2O (see Note 5). The transcript can be checked by running 1 µL on a 10% polyacrylamide gel alongside RNA markers to give a rough estimation of the transcript concentration and size. Stain the gel with toluidine blue after the gel run.

3.3. Methylation of RNA by Erm(E)

1. Each methylation reaction contains 5 µL of the RNA transcript, 24 µL of buffer C containing 1 mM S-adenosylmethionine (AdoMet), and 1 µL of Erm(E). Incubate for 45 min at 30 °C (see Notes 7 and 8).
2. Reactions are stopped by extraction with 1 volume of phenol, then 1 volume of phenol/chloroform, and finally 1 volume of chloroform (as described in Step 4 of Section 3.2). Precipitate the RNA, wash the pellet with ethanol, and redissolve in 6 µL of H_2O as described in Step 5 of Section 3.2.
3. The RNA samples are now ready for analysis by MALDI mass spectrometry (see Note 9).

3.4. Analysis of Methylation by MALDI Mass Spectrometry

1. The methylated RNA substrates are to be digested with RNase A or RNase T1 to produce oligonucleotides of a size suitable for MALDI mass spectrometry analysis (see Note 10). Samples of rRNA (2.5 pmol) are mixed with 0.5 µL of 3-HPA (0.5 M in 50% acetonitrile), 1 µg of RNase A, or 10 units of RNase T1 (see Note 11) and H_2O to a final volume of 5 µL and are digested for 2.5 h at 37 °C.
2. Purify the digested fragments on Poros 50R3 columns (see Note 12) as follows: A GelLoader tip (Eppendorf) is flattened at the tip of the extended outlet with forceps, reducing the inner diameter to less than 50 µm. 50 µL of methanol are filled into the GelLoader tip, and 1 µL of the precipitate of Poros 50R3 material in methanol is added. Mount the GelLoader tip onto a 1-mL syringe, and press the methanol through to form a column. The column is washed once with 50 µL of methanol and equilibrated by adding 50 µL of 10 mM triethyl ammonium acetate (TEAA), pH 7.0. Load the RNA digestion in 0.3 M TEAA onto the microcolumn, and wash with 50 µL of 10 mM TEAA and elute with 10 µL of 10 mM TEAA/25% acetonitrile (see Note 13). Dry the eluate and dissolve in water to 1–2 pmol/µL.
3. Prepare samples for MALDI mass spectrometry by mixing on the target 1 µL of the purified RNA digest, 0.5 µL of 3-HPA matrix (see Note 14), and a small volume (˜0.1 µL) of the ammonium-loaded cation exchange material. Leave to air-dry, and remove as much cation exchange material as possible under a microscope using a pipette tip. Sample preparation for MALDI tandem mass spectrometry is performed the same way.
4. The oligonucleotides are analyzed using a Voyager STR MALDI mass spectrometer (Applied Biosystems) in delayed extraction reflector time-of-flight mode detecting

positive ions (see Note 15). Spectra can be smoothed and calibrated using the Data Explorer software supplied with the mass spectrometer (see Note 16).

5. The exact nucleotide position of a modification can be located by tandem mass spectrometry ***(23)***. This can be carried out on a MicroMass MALDI Q-TOF Ultima mass spectrometer in positive ion mode (see Note 17). Generally, the window for parent ion selection is set at 2 *m/z* units, and the collision energy varies between 40 and 100 eV, depending on the mass of the parent ion. When required, spectra can be smoothed and calibrated using the MassLynx software supplied by the manufacturer.

4. Notes

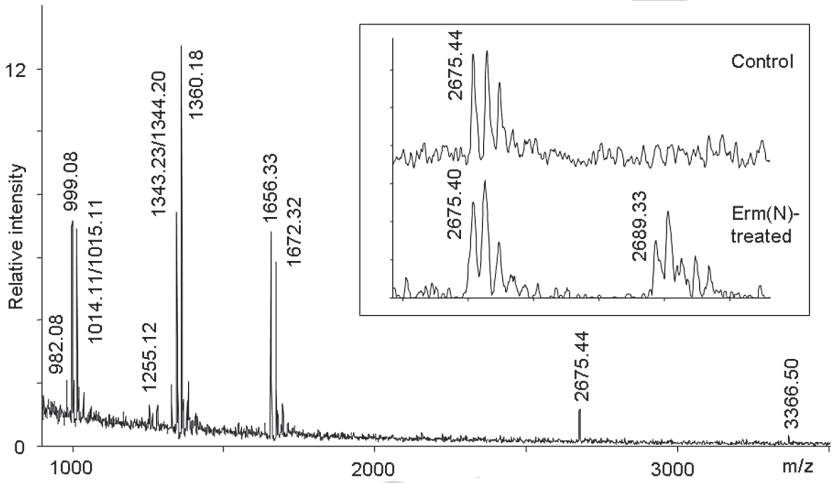
1. The entire *erm(E)* gene from *Saccharopolyspora erythraea*, the producer of the macrolide antibiotic erythromycin, has been cloned into R1-derivative plasmids such as pJEK47 ***(21)*** and pJEK48 ***(33)***. These plasmids are suitable for expression of large amounts of active Erm(E) in *E. coli*.
2. With its overall length of 385 amino acids, Erm(E) is significantly longer than most other members of the Erm family. Alignments with other Erm methyltransferases ***(24,27)*** show that they are generally 10 to 35 amino acids shorter at their N-termini and approximately 90 amino acids shorter at their C-termini. In one recombinant version of Erm(E), expressed from plasmid pSDdiv ***(26)***, we removed the C-terminal 89 amino acids and added a histidine-tag without any apparent loss of methylation activity (see Note 4).
3. The Erm(E) methyltransferase associates with ribosomes under low- to moderate-salt conditions (here, up to 100 mM NH_4Cl) and is released by washing with a high-salt buffer (buffer A with 1 M NH_4Cl) ***(20,21)***. The solubility of Erm(E) is reduced in the absence of monovalent ions (buffer B), but the enzyme becomes readily soluble again on increasing the salt concentration (buffer C).
4. Fairly high Erm(E) purity (about 80%) is achieved by this procedure. A higher purity (>95%) can be obtained on Ni-NTA resin (Qiagen) after a histidine-tag has been added to the C-terminal of Erm(E). The activity of Erm(E) and other orthologs we have tried, such as Erm(N), remain unaffected by a C-terminal histidine-tag. However, we observed a reduction in Erm(E) activity with N-terminal tags ***(34)***.
5. It is important not to excessively dry nucleic acid pellets, as they can be difficult to redissolve. DNA and RNA pellets are best dried by removing all visible traces of 70% ethanol using a micropipette and then leaving the tubes with the lids open on the bench at ambient temperature for 30 min to allow any remain traces of ethanol to evaporate.
6. Slightly higher yields of *in vitro* transcripts can be obtained by extending the incubation up to a maximum of 16 h (overnight). Longer times yield diminishing returns, probably due to RNA breakdown.
7. As discussed in the Introduction, members of the Erm family are either mono- or dimethyltransferases, and addition of two methyl groups to the A2058 target by

the latter type confers the more severe resistance phenotype. All Erm methyltransferases have but a single AdoMet binding site, and thus dimethylation proceeds in a two-step manner ***(35)*** requiring recharging of the enzyme with a new cofactor molecule. Under both *in vivo* and *in vitro* conditions, Erm(E) is an extremely effective dimethyltransferase adding the second methyl group very rapidly, and it is rare that we find a trace of the monomethylated RNA intermediate. Other Erm dimethyltransferases we have studied, including the streptococcal Erm(B) ***(36)*** and the mycobacterial Erm(38) ***(37)***, are distinctly less efficient at adding the second methyl group to A2058. The effects of Erm dimethyltransferase inhibitors might first become evident as an accumulation of monomethylated product. In Fig. 2, we have used a recombinant version of the monomethyltransferases Erm(N) (histidine-tagged at the C-terminus; see Note 4) to demonstrate MALDI-MS detection of the monomethylated product.

8. Potential methyltransferase inhibitors can be added in buffer C, while maintaining the total volume reaction at 30 µL of buffer C. It will be necessary to stop reactions at a series of time points if IC_{50} values of potential inhibitors are to be estimated.
9. A rapid and accurate estimate of A2058 dimethylation can be obtained by primer extension with reverse transcriptase ***(21)***. Primer extension requires appreciably less RNA than MALDI mass spectrometry (about 10% the amount) but has the disadvantage that it cannot detect monomethylation at the N^6 of A2058.
10. Masses of the RNase digestion fragments can be calculated using the Mongo Oligo Mass Calculator (http://www-medlib.med.utah.edu/masspec/mongo.htm). Digestion products smaller than trinucleotides are unsuited for MALDI time-of-flight mass spectrometry, because the lower *m/z* range is crowded with numerous signals including those from the matrix and buffers ***(31)***.
11. The RNase T1 digestion products will predominantly harbor 2´-3´ cyclic phosphates under the conditions described here. Increasing the digestion time or enzyme concentration will result in a greater proportion of digestion products with a 3´-phosphate ***(31)***; these are heavier by 18.01 Da (monoisotopic mass).
12. Commercial cartridges ready-packed with reverse-phase chromatography material are available from various suppliers including Waters (ZipTip cartridges) and Proxeon (StageTip cartridges).
13. Size fractionation of digestion fragments can be obtained by stepwise elution with increasing concentrations of acetonitrile ***(38)***; all the fragments (Figure 2B and 2C) will be eluted by 25% acetonitrile.
14. Other matrices that yield higher sensitivity and/or resolution have been reported for oligonucleotide analysis by MALDI mass spectrometry ***(39,40)***. However, we prefer the 3-HPA matrix for this type of application, because it discriminates less between digestion fragments, i.e., nearly all fragments are detected regardless of their nucleotide composition or sequence.

15. The resolution of the delayed extraction, reflector time-of-flight mass analyzer is required to resolve the approximately 1 Da mass difference between U- and C-nucleotides. The instrument may also be operated in negative ion mode with

(a)



(b)

RNase A fragment	MH^+	Sequence
115-117	982.16	AAC
16-18	999.14	GAU
53-55	999.14	AGU
123-125	999.14	GAU
8-10	1014.15	GGC
22-24	1014.15	GGC
28-30	1014.15	GGC
86-88	1014.15	GGC
58-60	1015.14	GGU
1-3	1255.04	pppGGU
118-121	1343.21	AGGC
129-132	1344.19	GGAU
32-35	1360.19	GGGU
74-77	1360.19	GGGU
38-42	1656.26	AGAAC
89-93	1656.26	AAGAC
48-52	1672.26	GAGAC
94-101	***2675.41***	***GGAAAGAC***
105-114	3366.49	GAAAGGGGAU

Fig. 2. *(continued)*

(c)

RNase T1 fragments	MH^+	Sequence
116-118	879.17	AUC-OH
17-19	975.13	UCG
27-29	975.13	CUG
41-43	975.13	UCG
79-81	998.16	ACG
14-16	999.14	AUG
52-55	1281.16	UUCG
37-40	1327.21	AACG
48-51	1327.21	ACAG
75-78	1327.21	CAAG
83-86	***1351.22***	***AAAG***
21-25	1586.20	CUUCG
32-36	1611.19	UUUAG
67-72	1891.24	CCUUCG
7-13	2219.31	CACCUCG
108-114	2242.34	CACACCG
100-106	2291.34	AUAACAG
87-95	2853.40	ACCCCUACG
57-66	3137.38	UCCCUAUCUG

Fig. 2. (a) MALDI mass spectrum of fragments derived from RNase A digestion of structure A (Fig. 1). The fragment containing the A2058 target nucleotide (GGAAAGAC) runs at *m/z* 2675.40 (monoisotopic mass; see expanded region in box, and Note 18). Detection of a single methyl group is illustrated by the new signal 14 Da larger that appears in samples treated with the monomethyltransferase Erm(N); the methylation reaction with Erm(N) was stopped before it had run to completion. (b) Theoretical monoisotopic masses of the singly protonated RNase A fragments match the empirical masses to within 0.1 Da (see Note 10). All digestion fragments have 5´-OH and 3´-phosphate unless otherwise noted. The 94-101 fragment (italics) corresponds to the 23S rRNA nucleotides 2056 to 2063 and harbors the Erm target at A2058. (c) Theoretical monoisotopic masses of the singly protonated RNase T1 fragments derived from structure B (Fig. 1). The AAAG fragment at 83-86 (italics) corresponds to 23S rRNA nucleotides 2058 to 2061. This fragment is unique in structure B (but not in structure A) and gives a better resolved and more intense MS peak than the larger RNase A fragment containing nucleotide A2058.

similar or even better sensitivity, but instrument stability may be compromised by ion polarity switching if the ion source is contaminated.

16. We first perform an external calibration using appropriate oligodeoxynucleotides; this generally decreases the mass error below 0.5 Da. An internal calibration of the spectrum is subsequently performed using RNase digestion fragments, which are not modified by the methyltransferase.
17. Descriptions of the fragmentation behavior of singly protonated RNA in a MALDI Q-TOF instrument can be found in ***(41)***. The predominant sequence ions are of the

c- and y-type; for the nomenclature of nucleic acid fragment ions, see McLuckey et al. ***(42)***.

18. The natural isotopic distribution of ^{12}C and ^{13}C (approximately 99:1) leads to multiple signals at 1 Da increments, and this is visible upon closer inspection of the MS peaks. The multiplicity is more pronounced for larger oligonucleotide fragments due to the binomial distribution of the carbon isotopes.

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