Supplementary Figures

A single methyltransferase YefA (RlmCD) catalyzes both m^{5}U747 and m^{5}U1939 modifications in *Bacillus subtilis* 23S rRNA

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Figure S1: MALDI tandem mass spectrum of *B. subtilis* 23S rRNA fragment containing m^{5}U747

Analysis of this nucleotide was facilitated by generating partial RNase T1 digestion products from the G725 to G772 region in wild-type rRNA. The partial RNase T1 product CACGUUG>p corresponds to nucleotides 742 to 748 (box) and contains m^{5}U747. This fragment had an initial mass of 2256.3 *m/z* (MH$^+$). Generation of the ion pattern after further fragmentation by tandem MS is illustrated; the ions are annotated according to McLuckey et al 1992.

Figure S2: MALDI tandem mass spectrum of *B. subtilis* 23S rRNA fragment containing m^{5}U1939

This nucleotide was analysed after generation of partial RNase A digestion products from the C1914 to C1961 region in wild-type rRNA. The partial RNase A product GAAAUU>p corresponds to nucleotides 1935 to 1940 (box) and contains m^{5}U1939. This fragment had an initial mass of 1959.3 *m/z* (MH$^+$). The ion fragmentation pattern is annotated as in Fig. S1.

Figure S3: Amino acid sequence alignment of the m^{5}U methyltransferase YefA and its *B. subtilis* paralog YfjO

Identical amino acids are highlighted in black. Both proteins possess the N-terminal extension seen in RlmD and missing in RlmC.

**Figure S4: LC-MS^n analysis of the m^{5}U ribonucleoside from *B. subtilis* tRNAs**

(*A*) MS of the intact negatively charged m^{5}U nucleoside at *m/z* 257, which upon MS^2 fragmentation (*B*) yielding ions of *m/z* 124, 125, 167 and 214. (*C*) Further fragmentation of the *m/z* 214 ion (MS^3) produced ions of *m/z* 80 and 96. The structures of the main ions produced are shown, and their fragmentation pattern is unambiguously consistent with the initial uridine at *m/z* 257 being methylated on its C-5 atom.

Figure S5: HPLC profiles of tRNA nucleosides

(*A*) The digestion products of total tRNAs from the wild-type *B. subtilis* strain 168, highlighting the unmodified nucleosides. (*B*) Enlargement of the boxed region containing the m^{5}U modification. Enlargements of the same region for tRNA nucleosides from (*C*) *B. subtilis ΔyefA,* (*D*) *B. subtilis ΔyfjO,* (*E*) *E. coli* wild-type cells, (*F*) *E. coli* strain *ΔrlmC/ΔrlmD/ΔtrmA*. (*G*) Nucleoside standardization mixture that includes m^{5}U; the fraction corresponding to the retention time for m^{5}U is indicated.

Reference:

McLuckey, S. A., Van Berkel, G. J., and Glish, G. L. (1992) Tandem mass spectrometry of small multiply charged oligonucleotides, *J Am Soc Mass Spectrom* **3**, 60-70.
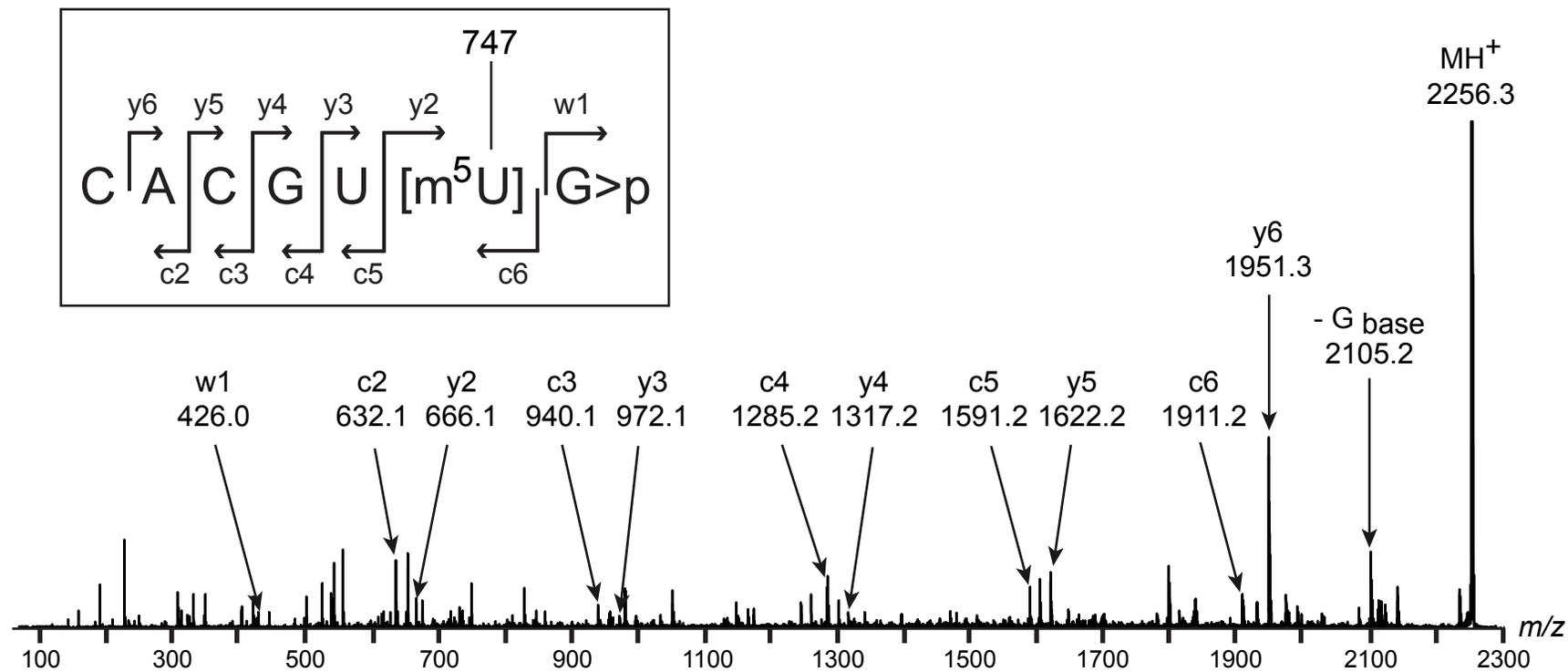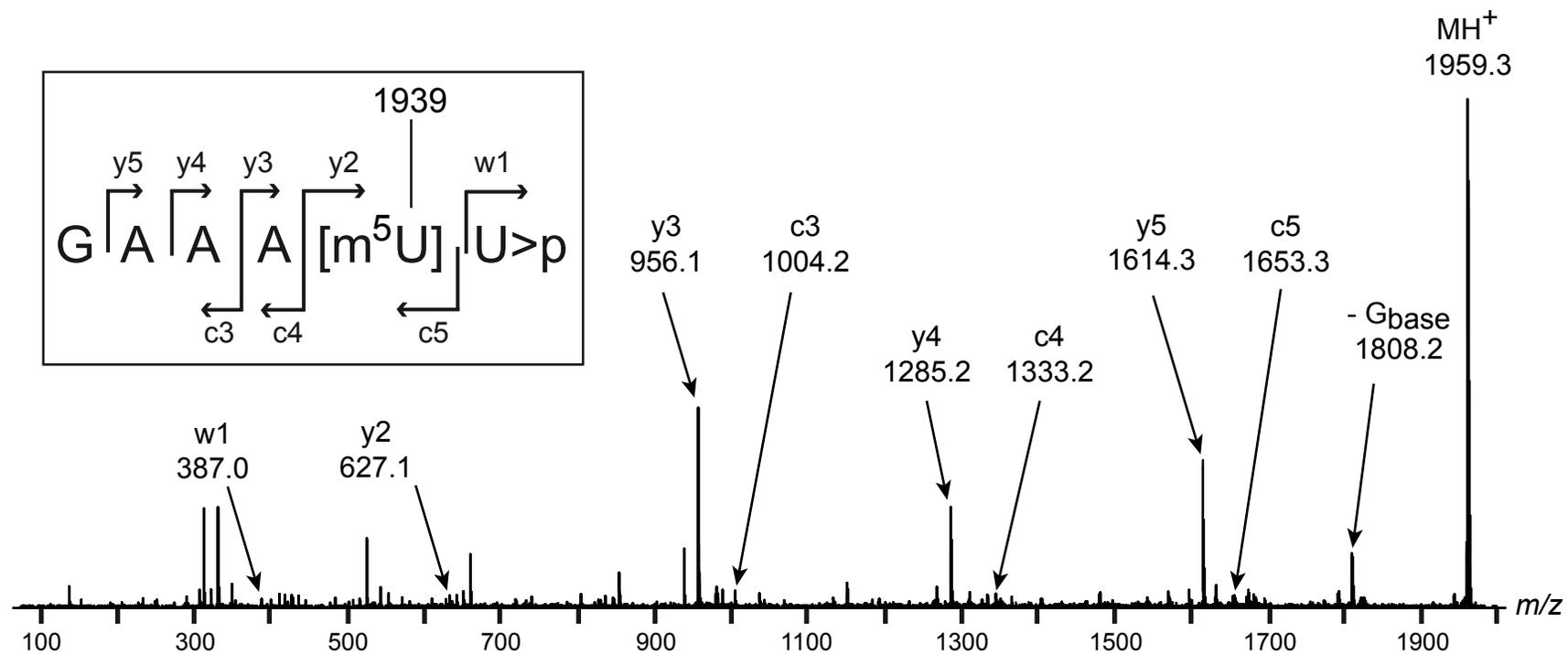
Figure S1

Figure S2

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YefA/    ---MKMKPPVEKN--EYYDVTFEDLTHEGAGVAKVQGFPIFVPNALPEEKAQIKVTRVKK
YfjO/    MNQQKKQAPVELKVGQTFPLTIKRLGINGEGVGYFKKKVVFVPGALPGEEVVVQATKVQP

YefA/    GFAFGRLIELKEESPHRTDAPCPIYKQCGGCQLQHMTYEGQLLFKQKQVKDVLERIGKLD
YfjO/    KFSEGRIKKIRKASEHRVAPPCPVYEQCGGCQLQHLAYSQQLREKRDIVIQSLERHTKFK

YefA/    LSKVTVHPTLGMEDPWNYRNKAQVPVG-EREGGLVAGFYQQRSHDIIDMSACLIQQSKND
YfjO/    VENMEIKETIGMDNPWNYRNKSQFQIGRSQSGSIIAGLYGLDSHDIVPIKDCIVQHPATN

YefA/    EAVQAVKDICANYGVKAYNEERHKGWLRHIMVRYGVVTGEMMIVFITRTSDFPHKAKIIE
YfjO/    KTTGIVRRILEDFNVSVYNERKRKGDVRTIVTRVGFETGEVQVVLVTAKETLPHKEEIVK

YefA/    DITAQFPHVKSIVQNINPNKTNVIFGNETNVIWGEEYIYDLIGDVKFAISARSFYQVNPE
YfjO/    AIQKRLPEVKSIIQNVNGAKTSVIFGEKTKQLAGKTVIQEVLGDVSFELSARAFFQLNPE

YefA/    QTKVLYDKALEYAELQGEETVIDAYCGIGTISLFLAKQAKKVYGVEIVPEAIEDAKRNAE
YfjO/    QTVKLYDEVKKAAQLTGKEKVVDAYCGVGTIGMWVADGAKEVRGMDVIKESIDDAKKNAK

YefA/    LNGNTNAEFAVGEAETVIPKWYEEGITADTLVVDPPRKGCDEALLRTIVEMKPKRVVYVS
YfjO/    KHGMANATYVTGTAEHWLPKWTKEGFRPDVVIVDPPRTGCDSTFLDTIKKVKPKRFVYVS

YefA/    CNPGTLARDLRVLEDGGYVTREVQPVDMFPHTNHVECCVLIKLKE--
YfjO/    CNPSTLAKDLQTLSK-DYRVDYIQPVDMFPQTAHVEAVARLVLKSSN
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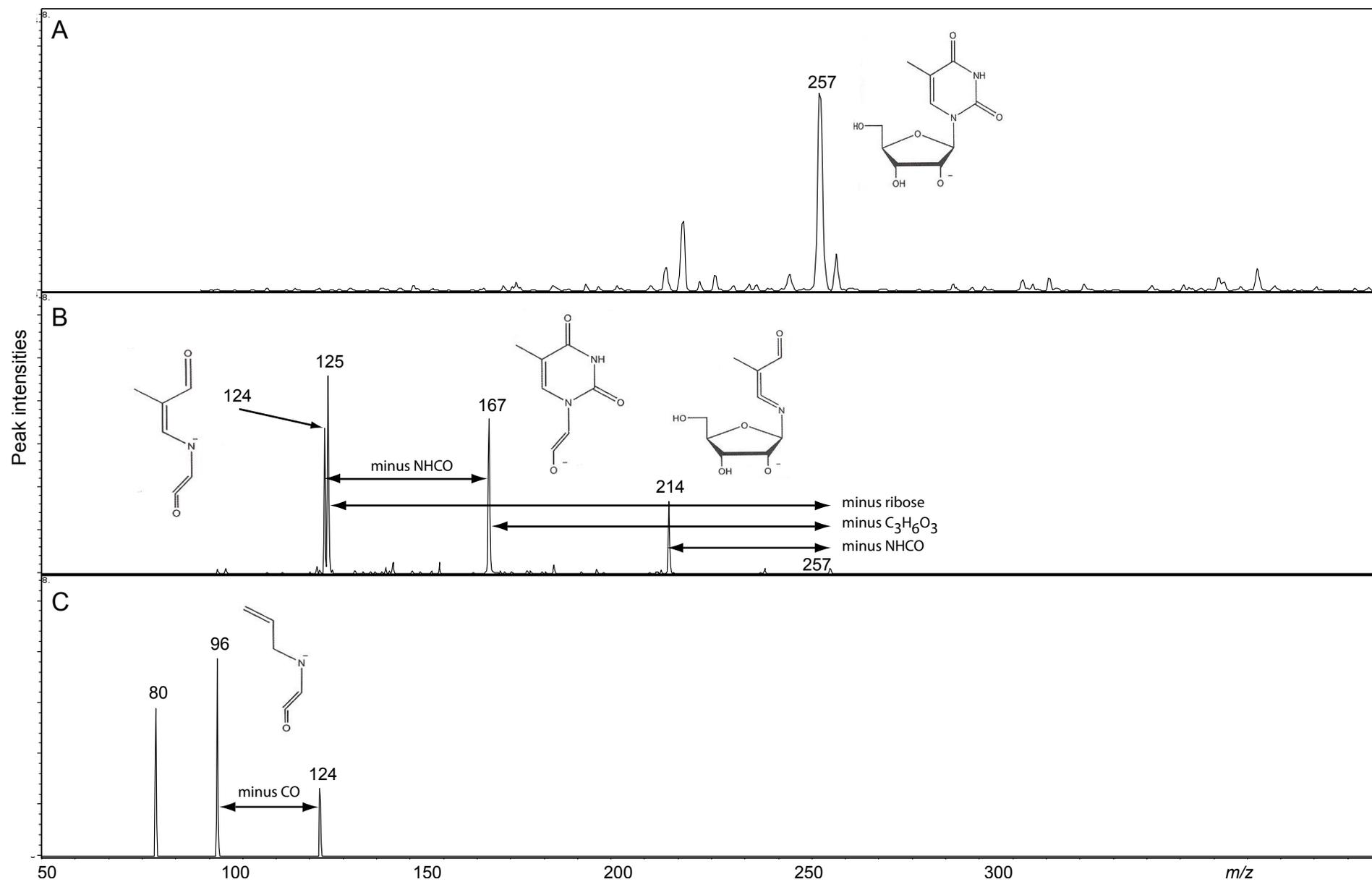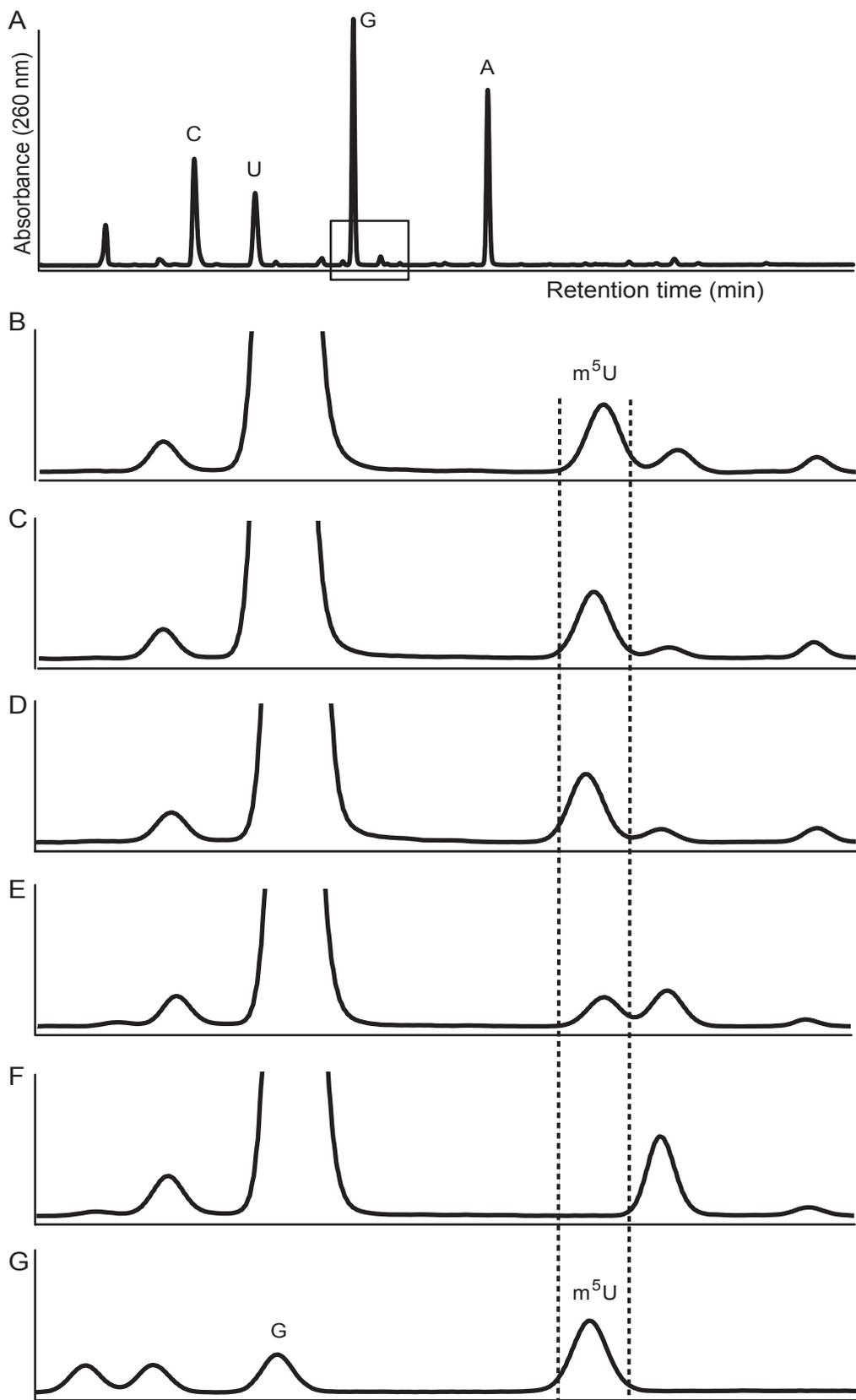
Figure S3

Figure S4

Figure S5