Electronic Supplementary Material (ESI) for RSC Chemical Biology.

Supporting Information

N^4-Allylcytidine: A New Nucleoside Analogue for RNA Labelling and Chemical Sequencing

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Section I: Characterizations of a^{4}C and a^{4}CTP and their synthetic intermediates

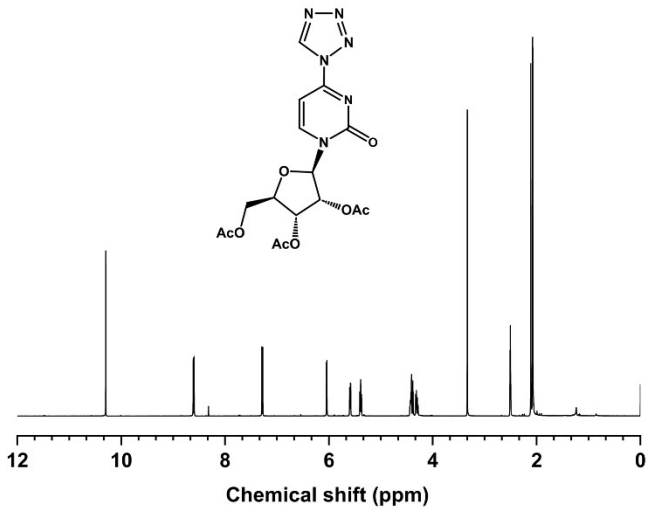


Figure S1. ^{1}H NMR spectrum of 4-(tetrazol-1-yl)-1-(2',3',5'-tri-*O*-acetyl-β-D-ribofuranosyl) pyrimidine-2-(1H)-one **(2)**. ^{1}H NMR (400 MHz, DMSO-d_6), δ (ppm): 10.30 (s, 1H), 8.60 (d, *J* = 7.3 Hz, 1H), 7.28 (d, *J* = 7.2 Hz, 1H), 6.04 (d, *J* = 3.4 Hz, 1H), 5.59 (dd, *J* = 3.3, 6.1 Hz, 1H), 5.39 (t, *J* = 6.5 Hz, 1H), 4.40 (d, *J* = 10.1 Hz, 2H), 4.27 – 4.35 (m, 1H), 2.11 (s, 3H), 2.08 (s, 3H), 2.07 (s, 3H).

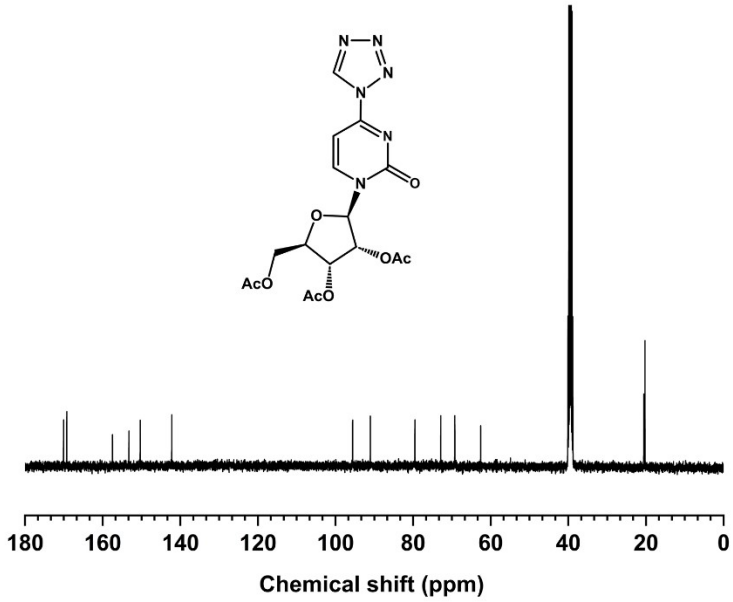


Figure S2. ^{13}C NMR spectrum of 4-(tetrazol-1-yl)-1-(2',3',5'-tri-*O*-acetyl-β-D-ribofuranosyl) pyrimidine-2-(1H)-one **(2)**. ^{13}C NMR (100 MHz, DMSO-d_6), δ (ppm): 170.1, 169.2, 157.5, 153.2, 150.3, 142.2, 95.6, 91.0, 79.5, 72.9, 69.3, 62.6, 20.6, 20.3

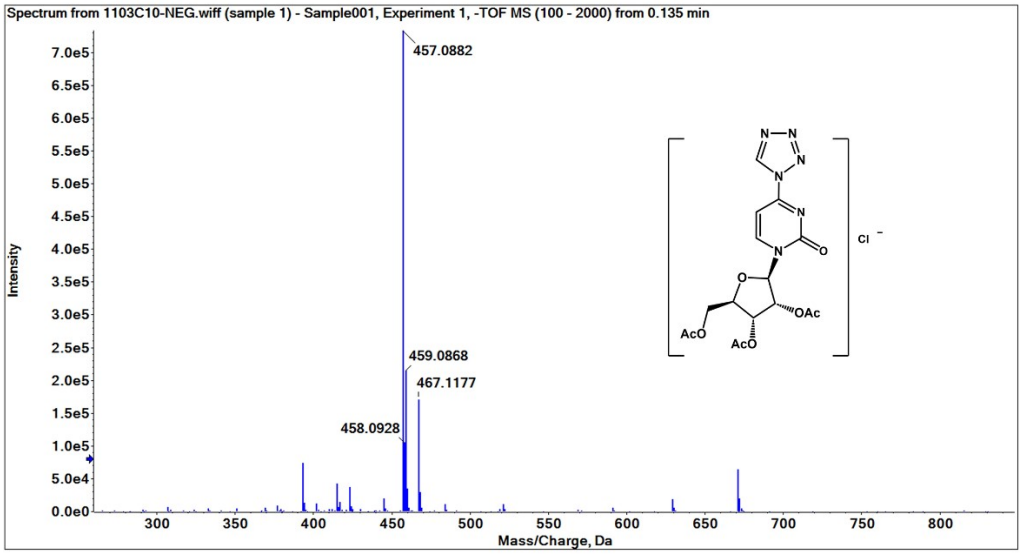


Figure S3. High-resolution mass spectrum (HRMS) of 4-(tetrazol-1-yl)-1-(2',3',5'-tri-*O*-acetyl-β-D-ribofuranosyl) pyrimidine-2-(1H)-one **(2)**. HRMS (ESI), *m/z* 457.0882 ([M+Cl]$^-$, calcd 457.0880).

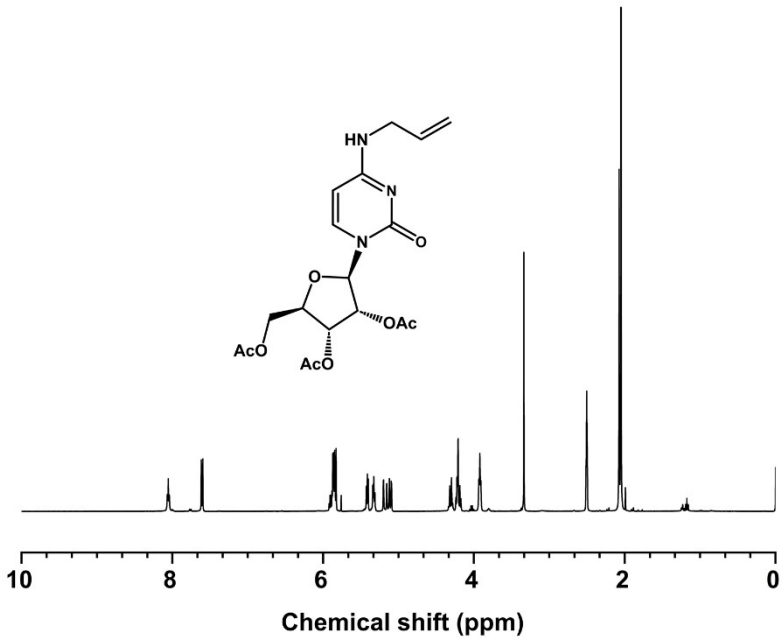


Figure S4. ^{1}H NMR spectrum of 2',3',5'-tri-*O*-acetyl-*N*^{4}-allylcytidine **(3)**. ^{1}H NMR (400 MHz, DMSO-d_6), δ (ppm): 8.05 (t, J = 5.6 Hz, 1H), 7.61 (d, J = 7.5 Hz, 1H), 5.76 – 5.97 (m, 3H), 5.37 – 5.47 (m, 1H), 5.33 (t, J = 5.9 Hz, 1H), 5.18 (dd, J = 1.7, 17.2 Hz, 1H), 5.11 (dd, J = 1.7, 10.2 Hz, 1H), 4.31 (d, J = 8.5 Hz, 1H), 4.13 – 4.26 (m, 2H), 3.86 – 3.98 (m, 2H), 2.07 (s, 3H), 2.05 (s, 6H).

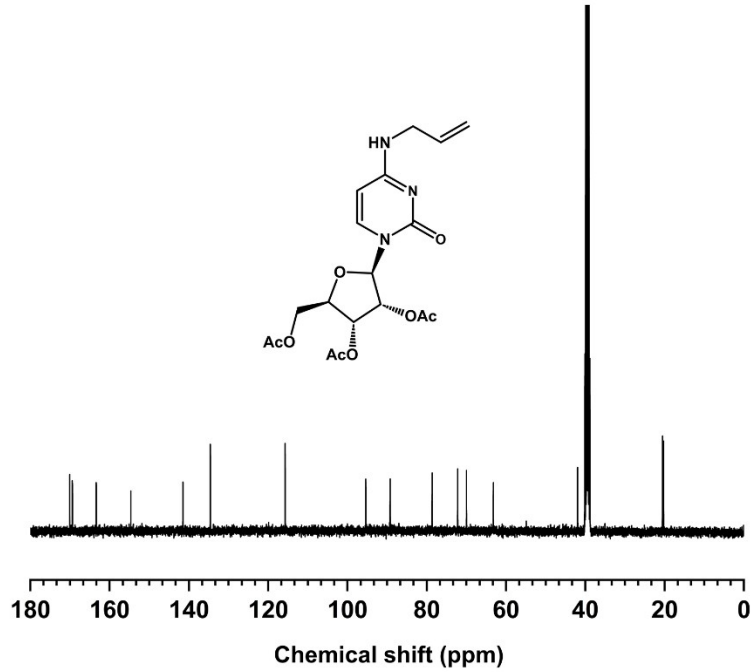


Figure S5. ^{13}C NMR spectrum of 2',3',5'-tri-*O*-acetyl-*N*^{4}-allylcytidine **(3)**. ^{13}C NMR (100 MHz, DMSO-d_6), δ (ppm): 170.1, 169.4, 169.3, 163.4, 154.6, 141.5, 134.6, 115.7, 95.4, 89.2, 78.6, 72.2, 70.0, 63.2, 42.0, 20.5, 20.3, 20.2

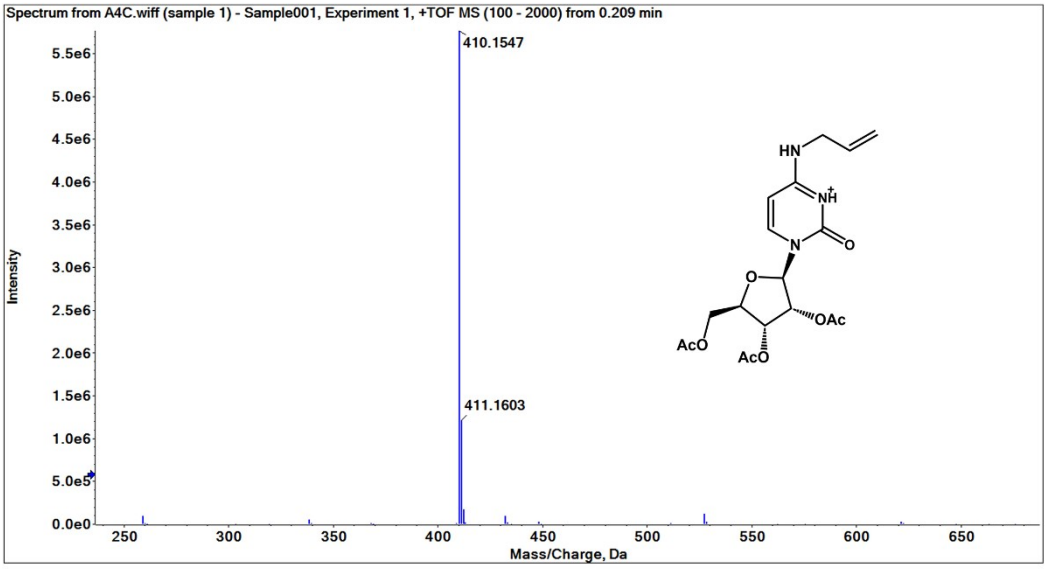


Figure S6. High-resolution mass spectrum of 2',3',5'-tri-O-acetyl-N^4-allylcytidine **(3)**. HRMS (ESI), *m/z* 410.1547 ([M+H]$^+$, calcd 410.1558).

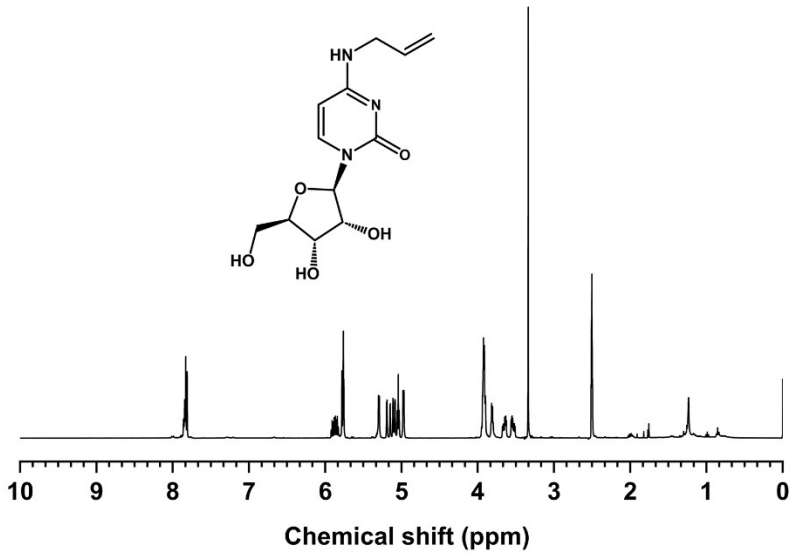


Figure S7. ^{1}H NMR spectrum of N^4-allylcytidine **(4)**. ^{1}H NMR spectrum (400 MHz, DMSO-d_6), δ (ppm): 7.74 – 7.90 (m, 2H), 5.87 (s, 1H), 5.76 (s, 2H), 5.30 (d, J = 5.2 Hz, 1H), 5.17 (dd, J = 1.8, 17.2 Hz, 1H), 5.10 (dd, J = 1.7, 10.2 Hz, 1H), 5.01 – 5.06 (m, 1H), 4.97 (d, J = 5.3 Hz, 1H), 3.92 (h, J = 4.4 Hz, 4H), 3.82 (t, J = 3.2 Hz, 1H), 3.64 (dt, J = 4.2, 8.4 Hz, 1H), 3.48 – 3.59 (m, 1H).

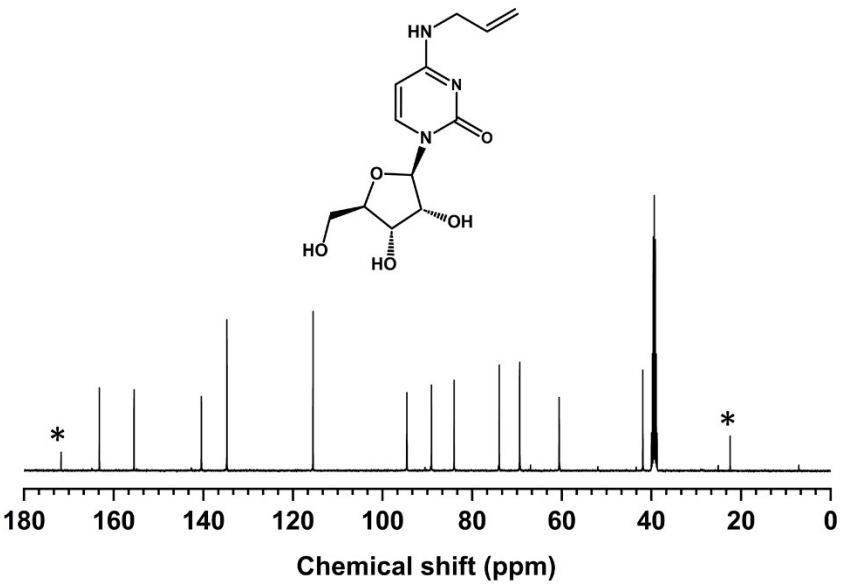


Figure S8. ^{13}C NMR spectrum of N^4-allylcytidine **(4)**. ^{13}C NMR spectrum (100 MHz, DMSO-d_6), δ (ppm): 163.2, 155.5, 140.4, 134.8, 115.5, 94.6, 89.1, 84.0, 74.0, 69.4, 60.6, 41.9. The asterisks (*) denote the signals from acetic acid.

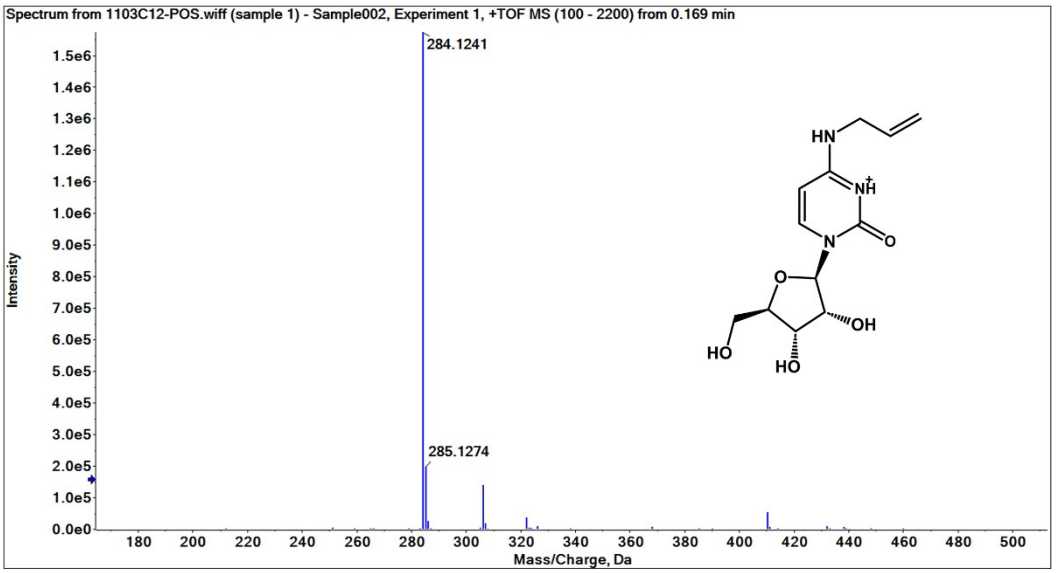


Figure S9. High-resolution mass spectrum of N^4-allylcytidine **(4)**. HRMS (ESI), *m/z* 284.1241 ([M+H]$^+$, calcd 284.1241).

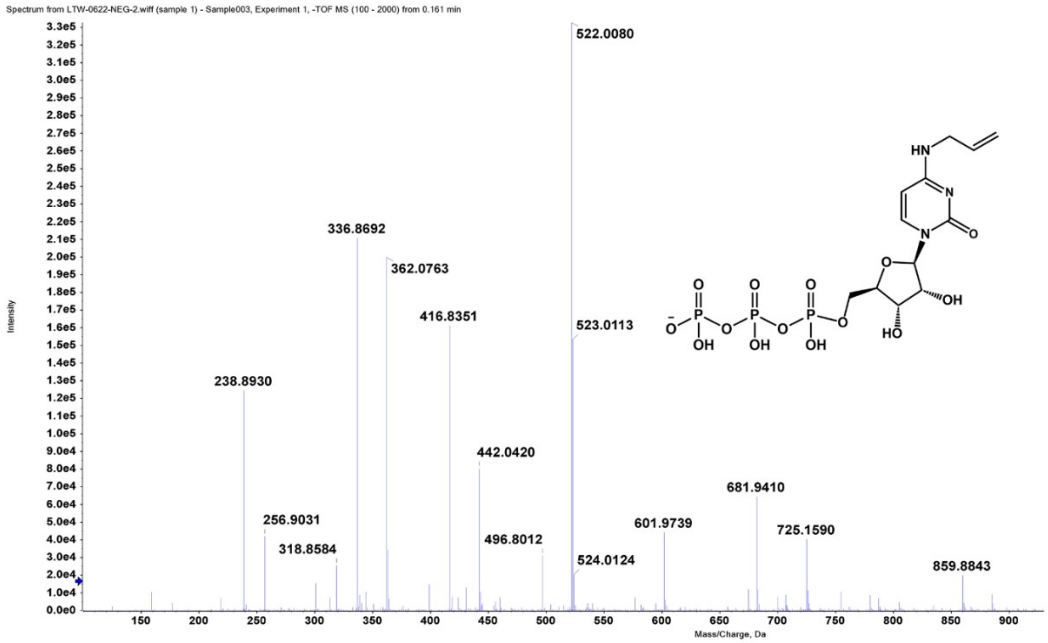


Figure S10. High-resolution spectrum of N^4-allylcytidine-5'-triphosphate **(5)**. HRMS (ESI), *m/z* 522.0080 ([M-H]$^-$, calcd 522.0085).

Section II: Model reactions of a^{4}C

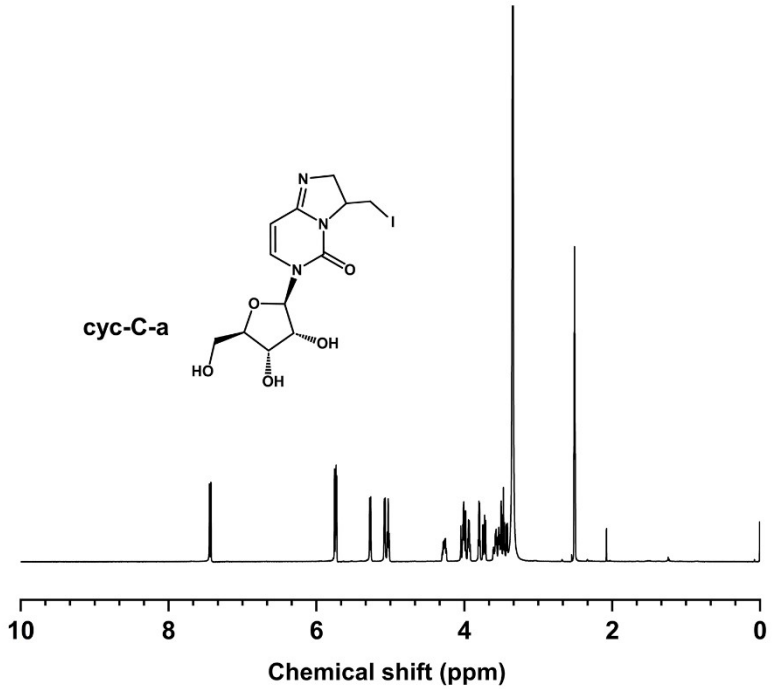


Figure S11. ^{1}H NMR spectrum (400 MHz, DMSO-d_6) of 3, N^4-cyclized cytidine (cyc-C-a). δ (ppm): 7.43 (d, J = 8.1 Hz, 1H), 5.80 – 5.66 (m, 2H), 5.26 (d, J = 6.3 Hz, 1H), 5.07 (d, J = 5.0 Hz, 1H), 5.02 (t, J = 5.1 Hz, 1H), 4.26 (dtd, J = 11.3, 5.7, 2.4 Hz, 1H), 4.06 – 3.96 (m, 2H), 3.96 – 3.90 (m, 1H), 3.79 (q, J = 3.4 Hz, 1H), 3.72 (dd, J = 10.1, 5.6 Hz, 1H), 3.61 – 3.48 (m, 2H), 3.48 – 3.39 (m, 2H).

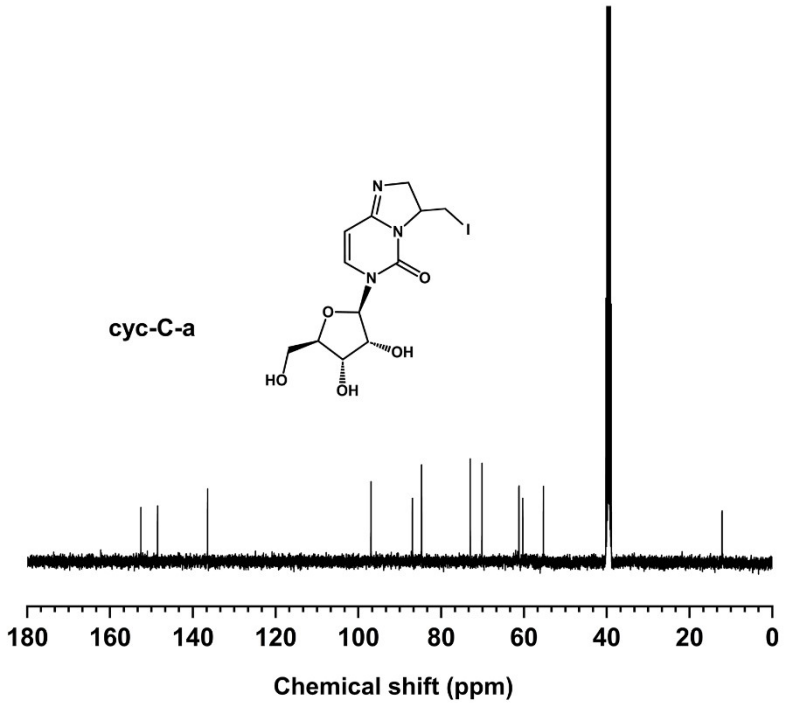


Figure S12. ^{13}C NMR spectrum (100 MHz, DMSO-d_6) of 3, N^4-cyclized cytidine (cyc-C-a). δ (ppm): 152.5, 148.5, 136.4, 96.9, 86.9, 84.7, 73.0, 70.1, 61.2, 60.3, 55.3, 12.1.

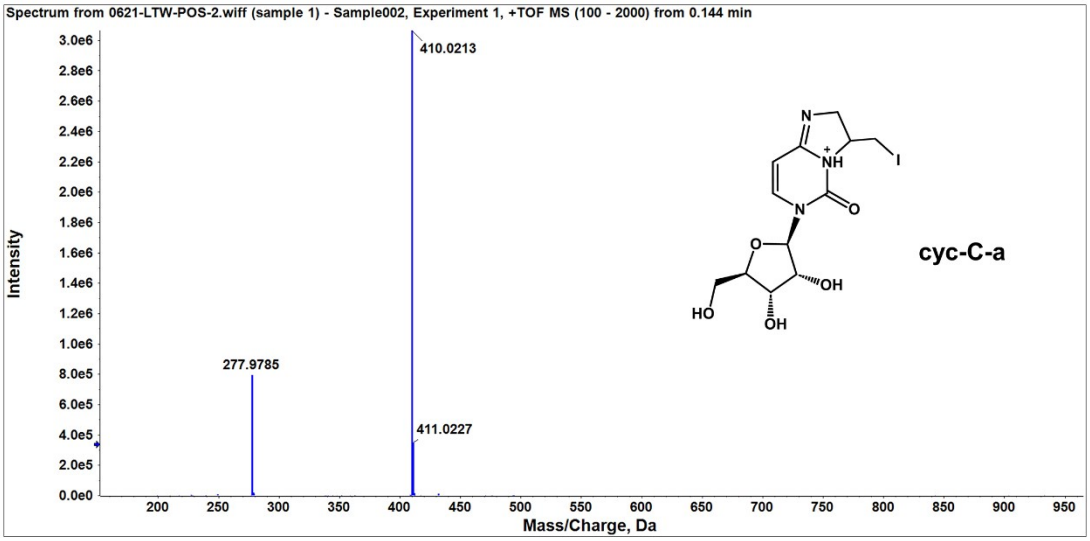


Figure S13. High-resolution mass spectrum of 3, N^4-cyclized cytidine (cyc-C-a). HRMS (ESI), *m/z* 410.0213 ([M+H]$^+$, calcd 410.0207).

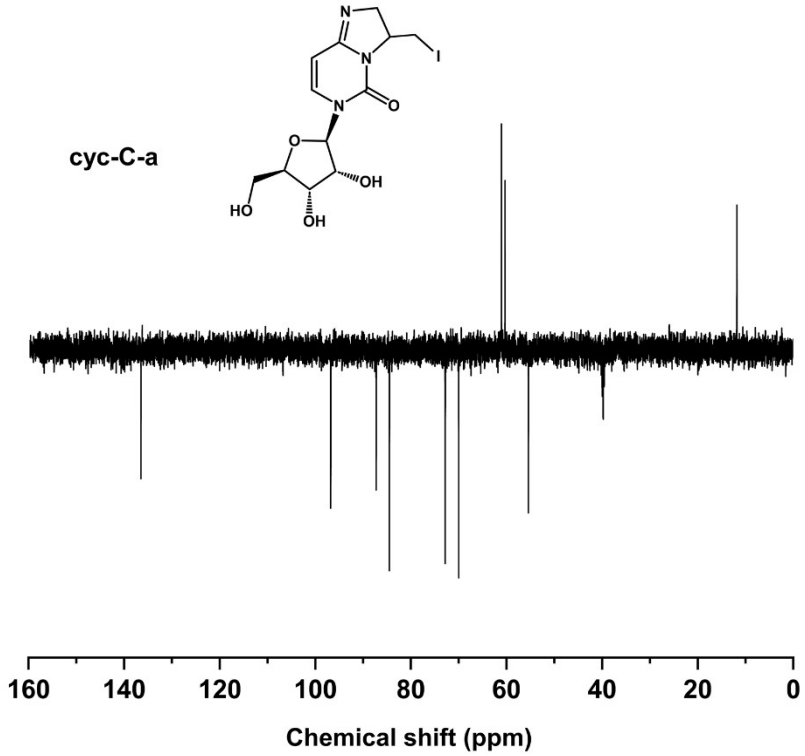


Figure S14. DEPT spectrum of 3, N^4-cyclized cytidine (cyc-C-a). δ (ppm): 135.9, 96.5, 87.0, 84.2, 72.6, 69.7, 60.8, 60.0, 55.2, 11.5

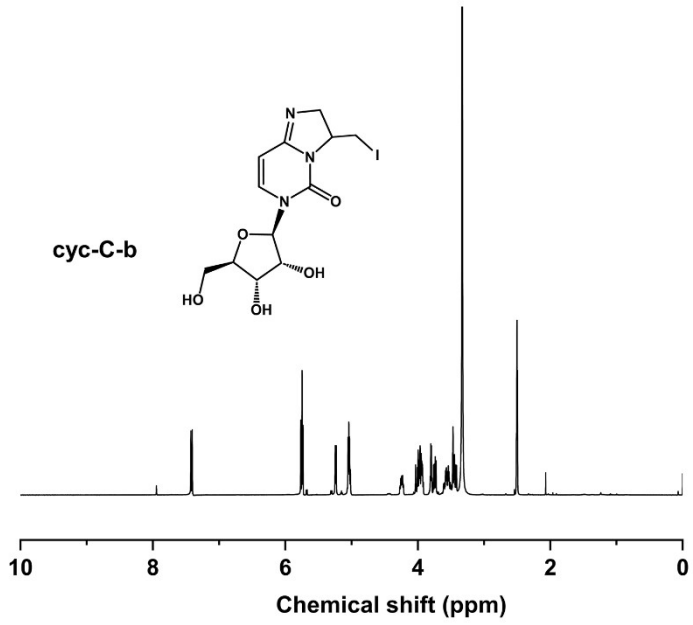


Figure S15. ^{1}H NMR spectrum (400 MHz, DMSO-d_6) of 3, N^4-cyclized cytidine (cyc-C-b). δ (ppm): 7.42 (d, J = 8.2 Hz, 1H), 5.84 – 5.69 (m, 2H), 5.24 (d, J = 5.8 Hz, 1H), 5.04 (p, J = 5.3 Hz, 2H), 4.25 (dtd, J = 10.6, 5.4, 2.1 Hz, 1H), 4.05 – 3.90 (m, 3H), 3.80 (q, J = 3.3 Hz, 1H), 3.75 (dd, J = 10.2, 5.3 Hz, 1H), 3.56 (qdd, J = 12.0, 5.1, 3.5 Hz, 2H), 3.47 (d, J = 2.3 Hz, 1H), 3.46 – 3.40 (m, 1H).

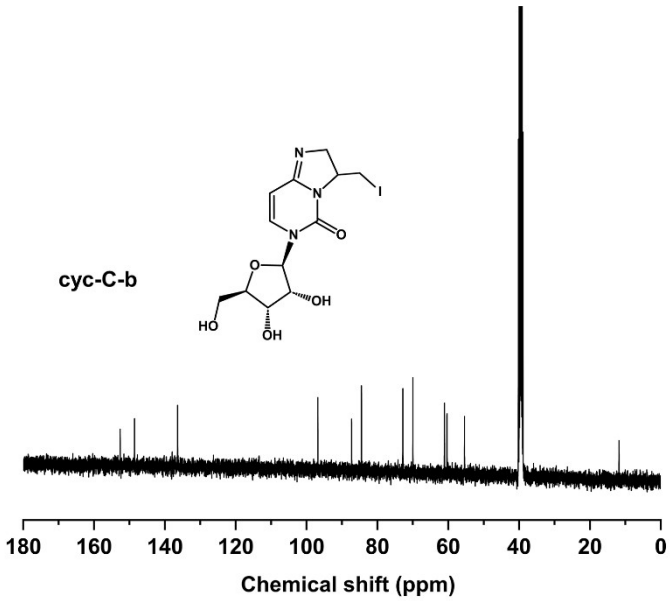


Figure S16. ^{13}C NMR spectrum (100 MHz, DMSO-d_6) of 3, N^4-cyclized cytidine (cyc-C-b). δ (ppm): 152.6, 148.5, 136.4, 96.8, 87.2, 84.4, 72.8, 70.0, 61.0, 60.3, 55.4, 11.7.

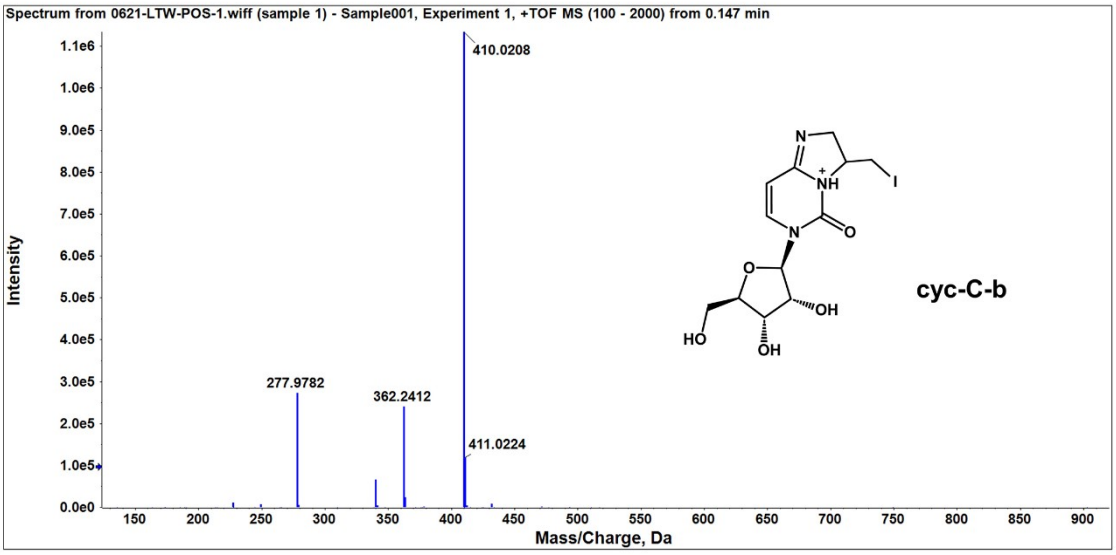


Figure S17. High-resolution mass spectrum of 3, N^4-cyclized cytidine (cyc-C-b). HRMS (ESI), *m/z* 410.0208 ([M+H]$^+$, calcd 410.0207).

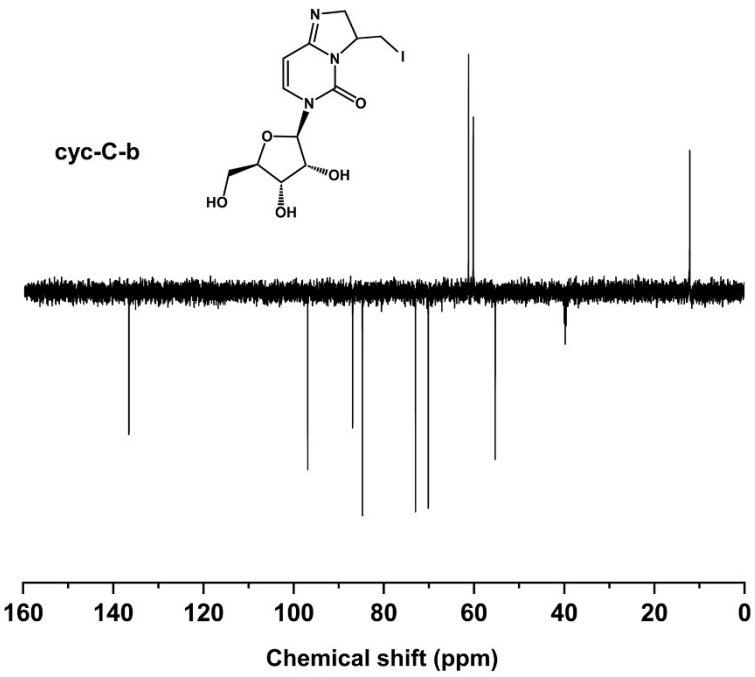


Figure S18. DEPT spectrum of 3, N^4-cyclized cytidine (cyc-C-a). δ (ppm): 136.3, 96.6, 86.7, 84.5, 72.7, 69.9, 60.9, 59.9, 55.1, 11.9.

Section III: Applications of a^{4}C in RNA sequencing

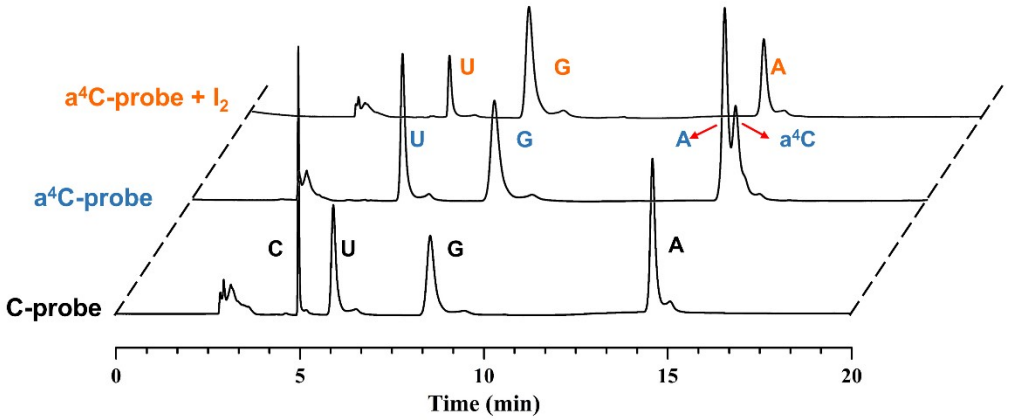


Figure S19. HPLC chromatograms of hydrolysed RNA C -probe,a^{4}C-probe and iodine-treated a^{4}C-probe .

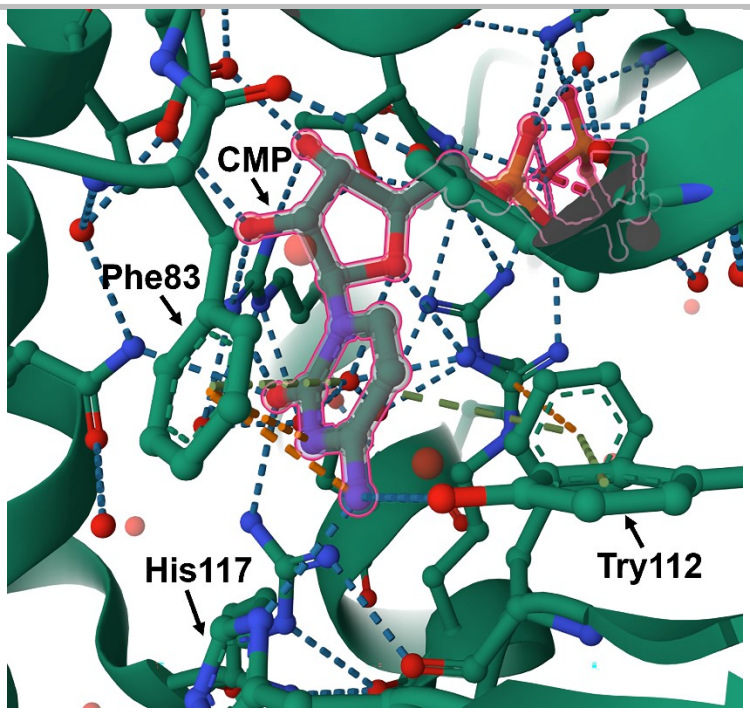


Figure S20. The X-ray crystal structure of UCK2 with the substrate cytidine monophosphate (CMP). This is adapted from the structure with PDB code 1XRJ (https://doi.org/10.2210/pdb1xrj/pdb).

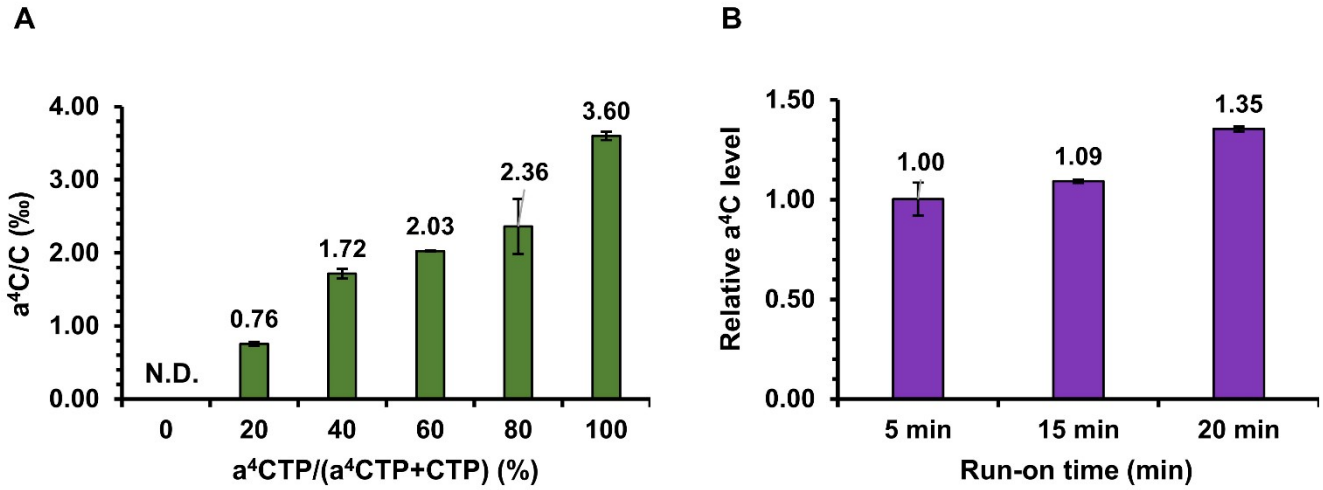


Figure S21. (A) The a^4C incorporation levels in RNA extracted from chromatin run-on reactions under different a^4CTP/CTP feeding ratios within 5 min. (B) The measured time course a^4C levels of RNAs extracted from chromatin run-on reactions in the presence of 100% a^4CTP.

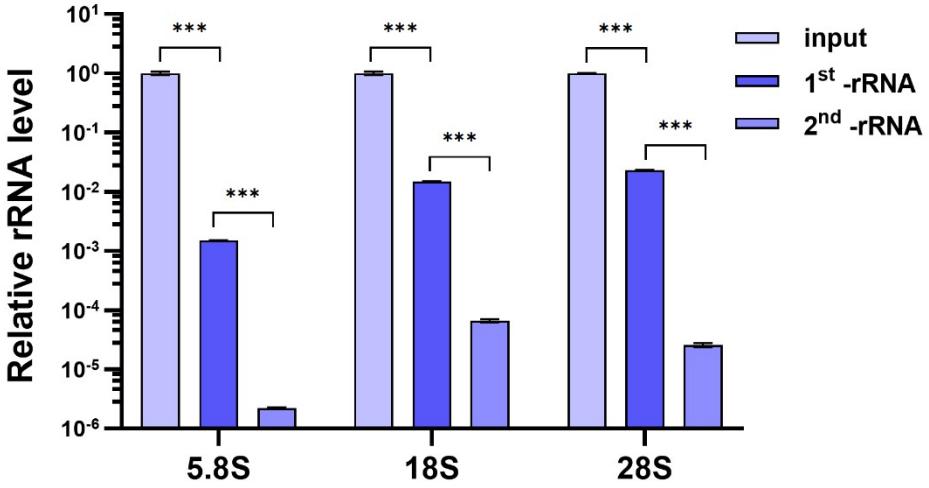


Figure S22. Quantification of different kinds of rRNAs after rRNA depletion by RT-qPCR.

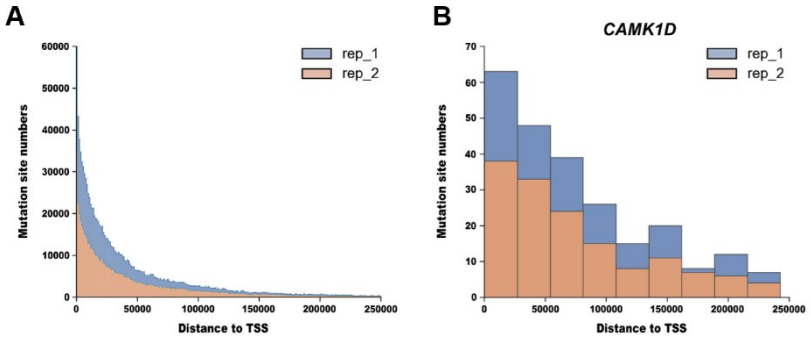
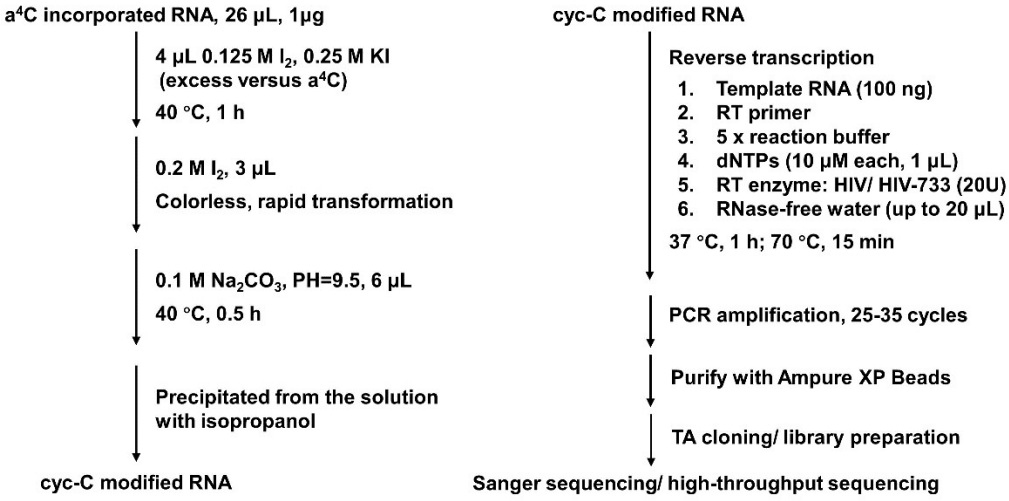


Figure S23. (A) The cumulative mutation site numbers (C-to-T) around RefSeq TSS by 50 bp-windows in a^{4}C-ChRO-seq using HIV 733 enzyme. (B) The cumulative mutation site numbers (C-to-T) of *CAMK1D* in a^{4}C-ChRO-seq around RefSeq TSS by 50 bp-windows.



Scheme S1. Flowchart of a^{4}C chemical sequencing in RNA.

Table S1. RNA probes used for *in vitro* chemical sequencing and enzyme-assisted labelling.IVT: in vitro transcription.

Name	Sequence (5'-3')	Length (nt)	Source	Purification
327nt-a^{4}C-probe	GGGAGATTAGGGTTAGGCGTTTTGCGCTGCTTCGCGATGTACGGGCCAGATATACGCGTT GACATTGATTATTGACTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCA TATATGGAGTTCCGCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACG ACCCCCGCCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTT CCATTGACGTCAATGGGTGGACTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTG TATCATATGCCAAGTACGCCCC	327	IVT	Zymo Research RNA-Clean& Concentrator
150nt-12S-mt-rRNA	GGGCTAAGAGTAGAGTGCTTAGTTGAACAGGGCCCTGAAGCGCGTACACACCGCCCGTCA CCCTCCTCAAGTATACTTCAAAGGACATTTAACTAAAACCCCTACGCATTTATATAGAGGA GACAAGTCGTAACATGGTAAGTGTACTGG	150	IVT	Zymo Research RNA-Clean& Concentrator

Table S2. DNA primers used in this work.

Name	Sequence (5'-3')	Purification
327-PCR-F	CGTAATACGACTCACTATAGGGAGATTAGGGTTAGGCGTTTTGCGCTG	PAGE
327-PCR-F	GGGGCGTACTTGGCATATGATACACTTGAT	PAGE
150-PCR-F	TAATACGACTCACTATAGGGCTAAGAGTAGAGTGCTTAGTTGAACAGG	PAGE
150-PCR-F	CCAGTACACTTACCATGTTACGACTTG	PAGE
GAPDH-qF	AGAAGGCTGGGGCTCATTTG	PAGE
GAPDH-qR	AGGGGCCATCCACAGTCTTC	PAGE
rRNA5.8S-qF	TTAGCGGTGGATCACTCGG	PAGE
rRNA5.8S-qR	GCAAGTGCGTTCGAAGTGTC	PAGE
rRNA18S-qF	GCGCGCAAATTACCCACTC	PAGE
rRNA18S-qR	CTCCAATGGATCCTCGTTAAAGG	PAGE
rRNA28S-qF	CCAGGGGAATCCGACTGTTTA	PAGE
rRNA28S-qR	CGTTTACCCGCGCTTCATTG	PAGE