Supporting Information

For

Existence of Internal *N*7-Methylguanosine Modification in mRNA Determined by Differential Enzyme Treatment Coupled with Mass Spectrometry Analysis

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The Supporting Information includes following items:

Preparation of DNA template for in-vitro transcription

PCR amplification was performed to generate the DNA template for in-vitro transcription. The PCR reaction mixture contained 0.25 μL of Ex Taq DNA polymerase (5 U/μL) (Takara, Dalian, China), 4 μL of dNTPs (2.5 mM for each), 1 μL of forward primer (10 μM, 5'-GAATTAATACGACTCACTATAGGGAGA-3'), 1 μL of reverse primer (10 μM, 5'-AGCCGTTTCTGTAATGAAGGAG-3'), 5 μL of 10 × Ex Taq buffer, 5 μL of the plasmid carrying the T7 promoter (10 ng/μL) and 33.75 μL of nuclease-free water. PCR reactions were performed as follows: 94°C for 2 min followed by 30 cycles at 94°C for 30 s, 56°C for 30 s and 72°C for 30 s, and finally at 72°C for 2 min. The PCR products were purified using E.Z.N.A.® Cycle-Pure Kit (Omega Bio-Tek Inc., Norcross, GA) according to the manufacture's recommended procedure. The purified DNA template was examined by 5% non-denaturing PAGE.

Enzymatic digestion conditions

As for S1 nuclease digestion, 0.5 μg of RNA (in 8.5 μL of H_2O) were firstly denatured by heating at 95^{o}C for 5 min and then chilling on ice for 2 min. After adding 1/10 volume (1 μL) of S1 nuclease buffer (30 mM CH_3COONa, pH 4.6, 280 mM NaCl, 1 mM $ZnSO_4$) and 90 units (0.5 μL) of S1 nuclease, the mixture (10 μL) was then incubated at 37^{o}C for 1 h.

As for phosphodiesterase I digestion, 0.5 μg of RNA (in 7 μL of H_2O) were firstly denatured by heating at 95^{o}C for 5 min and then chilling on ice for 2 min. After adding 1 μL of 10× alkaline phosphatase buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, pH 9.0) and 0.002 units (2 μL) of phosphodiesterase I, the mixture (10 μL) was then incubated at 37^{o}C for 1 h.

As for T4 DNA polymerase digestion, 0.5 μg of RNA (in 8 μL of H_2O) were firstly denatured by heating at 95°C for 5 min and then chilling on ice for 2 min. After adding 2 μL of 10× T4 DNA polymerase buffer (330 mM Tris-acetate, pH 7.9, 660 mM CH_3COOK, 100 mM $(CH_3COO)_2Mg$, 5 mM DTT) and 50 units (10 μL) of T4 DNA polymerase, the mixture (20 μL) was then incubated at 37°C for 12 h.

As for exonuclease I digestion, 0.5 μg of RNA (in 8 μL of H_2O) were firstly denatured by heating at 95°C for 5 min and then chilling on ice for 2 min. After adding 2 μL of 10× exonuclease I buffer (670 mM glycine-KOH, pH 9.5, 10 mM DTT, 67 mM $MgCl_2$) and 50 units (10 μL) of exonuclease I, the mixture (20 μL) was then incubated at 37°C for 12 h.

As for exonuclease T digestion, 0.5 μg of RNA (in 8 μL of H_2O) were firstly denatured by heating at 95°C for 5 min and then chilling on ice for 2 min. After adding 2 μL of 10× NEBuffer 4 (50 mM CH_3COOK, 20 mM Tris-acetate, pH 7.9, 10 mM $(CH_3COO)_2Mg$, 1 mM DTT) and 50 units (10 μL) of exonuclease T, the mixture (20 μL) was then incubated at 37°C for 12 h.

To the resulting solution of above enzyme digestion mixture, 4 μL of alkaline phosphatase buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, pH 9.0), 9 units (0.3 μL) of alkaline phosphatase, and 25.7 μL of H_2O were subsequently added. Then the incubation was continued at 37°C for an additional 30 min followed by adding 160 μL of H_2O and extraction with an equal volume of chloroform three times to remove protein.

The traditional enzymatic digestion was performed according to previous studies.[1-3] Briefly, 0.5 μg of RNA (in 8.5 μL of H_2O) were firstly denatured by heating at 95°C for 5 min and then chilling on ice for 2 min. After adding 1/10 volume (1 μL) of S1 nuclease buffer (30

mM CH_3COONa, pH 4.6, 280 mM NaCl, 1 mM $ZnSO_4$) and 90 units (0.5 μL) of S1 nuclease, the mixture (10 μL) was then incubated at 37°C for 2 h. To the resulting solution of above enzyme digestion mixture, 4 μL of alkaline phosphatase buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, pH 9.0), 9 units (0.3 μL) of alkaline phosphatase, 0.002 units (2 μL) of phosphodiesterase I, and 23.7 μL of H_2O were subsequently added. Then the incubation was continued at 37°C for an additional 2 h followed by adding 160 μL of H_2O and extraction with an equal volume of chloroform three times to remove protein.

The resulting aqueous layer was then collected and dried at 37°C under vacuum. The nucleosides mixture from RNA digestion was reconstituted in 65 μL of water and then analyzed by LC-ESI-MS/MS.

Table S1. The sequence of sense strand of DNA template for in-vitro transcription. Highlighted in red is the T7 promoter. Underlined is the first base for the in-vitro transcription. "p" represents the phosphate.

Name	Sequence (5' → 3')
Sense strand of DNA template	GAATTAATACGACTCACTATAGGGAGACAGACTAAACTGGCTG ACGGAATTTATGCCTCTTCCGACCATCAAGCATTTTATCCGTACT CCTGATGATGCATGGTTACTCACCACTGCGATCCCCGGGAAAAC AGCATTCCAGGTATTAGAAGAATATCCTGATTCAGGTGAAAATA TTGTTGATGCGCTGGCAGTGTTCCTGCGCCGGTTGCATTCGATT CCTGTTTGTAATTGTCCTTTTAACAGCGATCGCGTATTTCGTCTC GCTCAGGCGCAATCACGAATGAATAACGGTTTGGTTGATGCGA GTGATTTTGATGACGAGCGTAATGGCTGGCCTGTTGAACAAGTC TGGAAAGAAATGCATAAACTTTTGCCATTCTCACCGGATTCAGT CGTCACTCATGGTGATTTCTCACTTGATAACCTTATTTTTGACGA GGGGAAATTAATAGGTTGTATTGATGTTGGACGAGTCGGAATCG CAGACCGATACCAGGATCTTGCCATCCTATGGAACTGCCTCGGT GAGTTTTCTCCTTCATTACAGAAACGGCT
In-vitro transcribed 5'-m^7G-capped RNA	m^7G(5')ppp(5')GGGAGACAGACUAAACUGGCUGACGGAAUUUA UGCCUCUUCCGACCAUCAAGCAUUUUAUCCGUACUCCUGAUG AUGCAUGGUUACUCACCACUGCGAUCCCCGGGAAAACAGCA UUCCAGGUAUUAGAAGAAUAUCCUGAUUCAGGUGAAAAUAU UGUUGAUGCGCUGGCAGUGUUCCUGCGCCGGUUGCAUUCGA UUCCUGUUUGUAAUUGUCCUUUUAACAGCGAUCGCGUAUUU CGUCUCGCUCAGGCGCAAUCACGAAUGAAUAACGGUUUGGU UGAUGCGAGUGAUUUUGAUGACGAGCGUAAUGGCUGGCCUG UUGAACAAGUCUGGAAAGAAAUGCAUAAACUUUUGCCAUUC UCACCGGAUUCAGUCGUCACUCAUGGUGAUUUCUCACUUGA UAACCUUAUUUUUGACGAGGGGAAAUUAAUAGGUUGUAUUG AUGUUGGACGAGUCGGAAUCGCAGACCGAUACCAGGAUCUU GCCAUCCUAUGGAACUGCCUCGGUGAGUUUUCUCCUUCAUU ACAGAAACGGCU

Table S2. The MRM transitions and optimal parameters for the analysis of nucleosides by LC-ESI-MS/MS.

Analytes	Precursor ion	Product ion	DP/V	EP / V	CEP / V	CE / V	CXP / V
C	244.1	112.1	20.0	8.0	10.0	20.0	2.5
U	245.1	113.1	25.0	6.0	13.0	15.0	3.0
A	268.1	136.1	15.0	5.0	15.0	23.0	2.0
G	284.1	152.1	25.0	5.0	10.0	23.0	3.0
m^7G	298.1	166.1	20.0	10.0	15.0	20.0	3.0
DHzR	354.1	222.1	25.0	8.0	10.0	25.0	2.5

Figure S1. The prepared DNA template and synthesized 5'-m^{7}G-capped RNA. (A) Examination of DNA template by 5% non-denaturing polyacrylamide gel electrophoresis. Lane 1, DNA marker; lane 2, DNA template obtained by PCR amplification. (B) Examination of 5'-m^{7}G-capped RNA by 5% denaturing polyacrylamide gel electrophoresis. Lane 1, RNA marker; lane 2, synthesized 5'-m^{7}G-capped RNA.

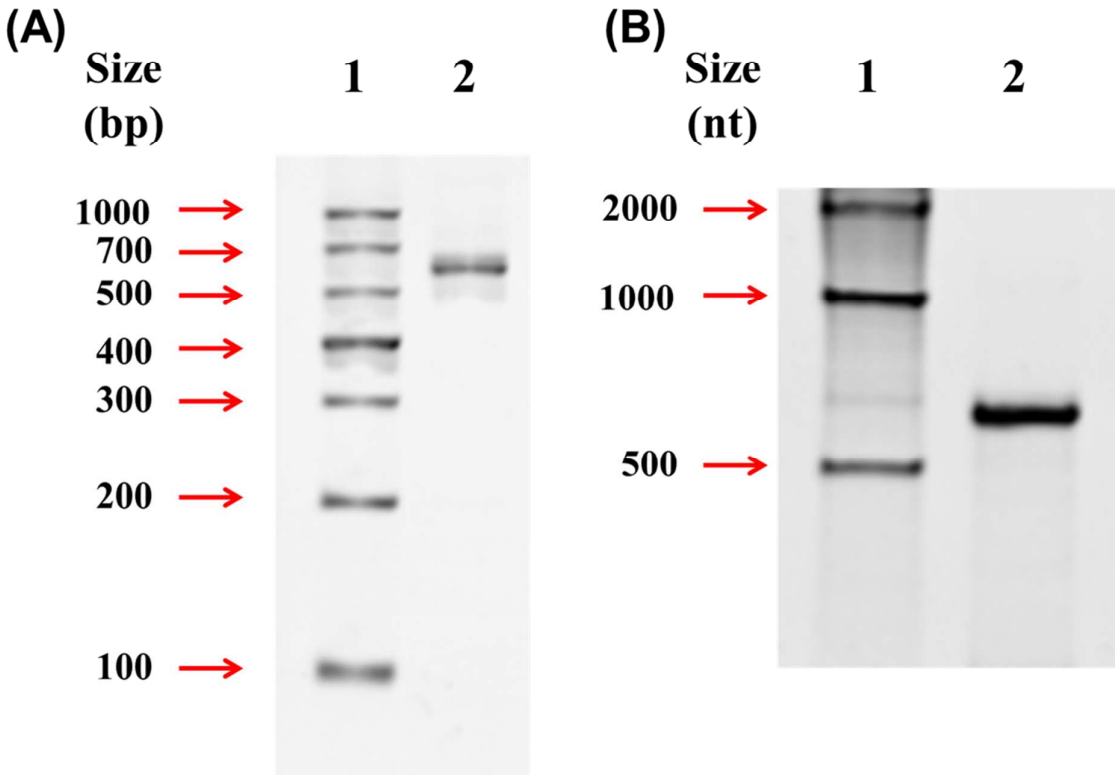


Figure S2. Optimization of digestion conditions. (A) Optimization of reaction time for S1 nuclease. (B) Optimization of reaction time for phosphodiesterase I. (C) Optimization of the concentration of S1 nuclease. (D) Optimization of the concentration of phosphodiesterase I.

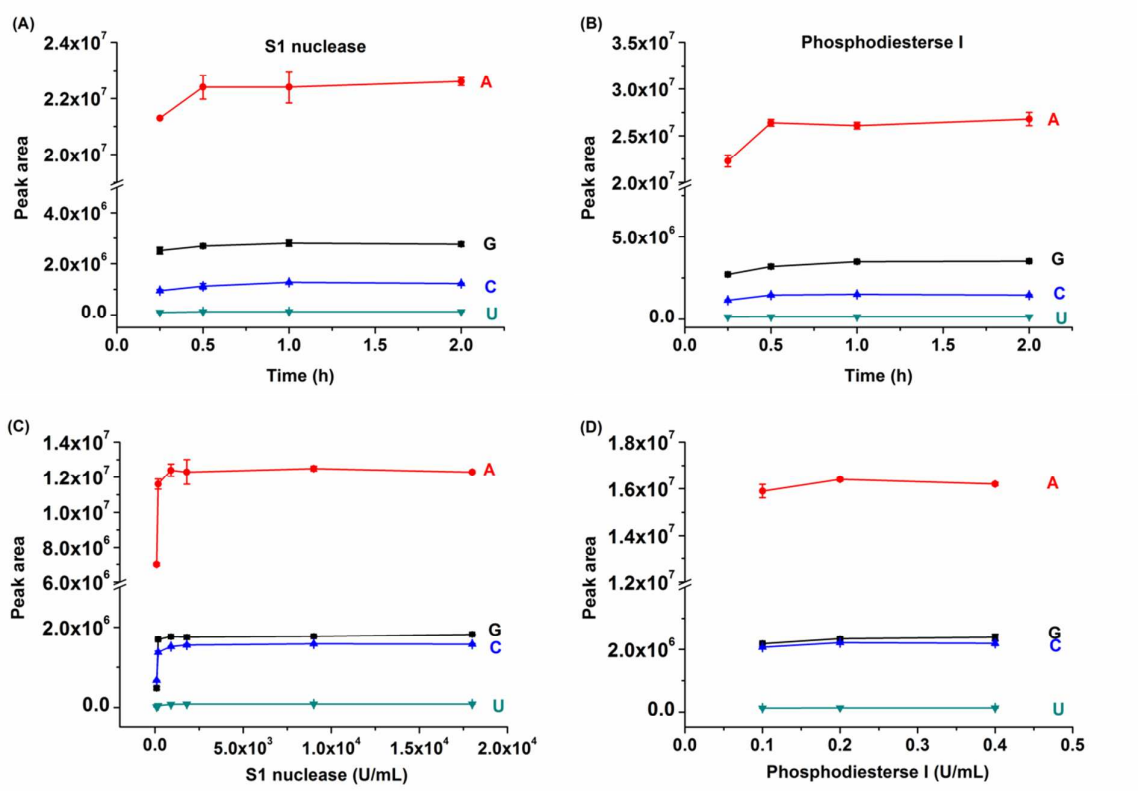


Figure S3. The calibration curves for quantification of m^7G. (A) 0.2-10 $m^7G/10^5$ G. (B) 10-200 $m^7G/10^5$ G.

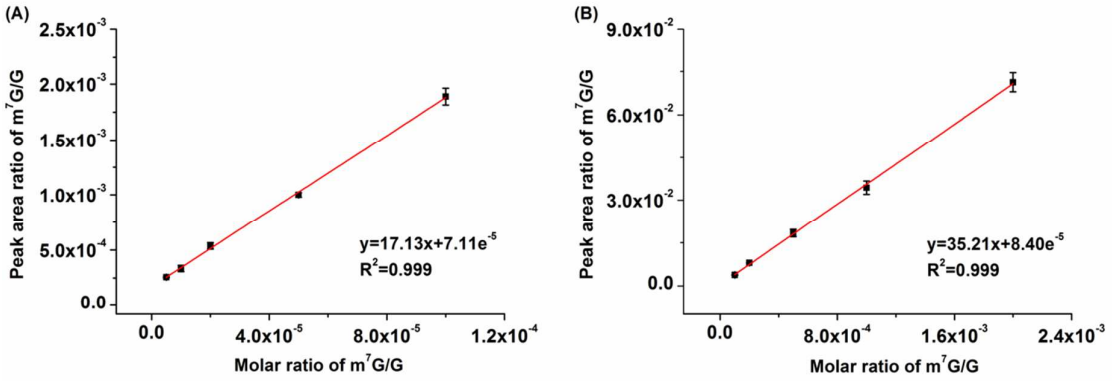


Figure S4. The influence of the amounts of substrate RNA on digestion specificity of S1 nuclease toward m^7G in the 5' cap of mRNA using the prepared 5'-m^7G-capped RNA.

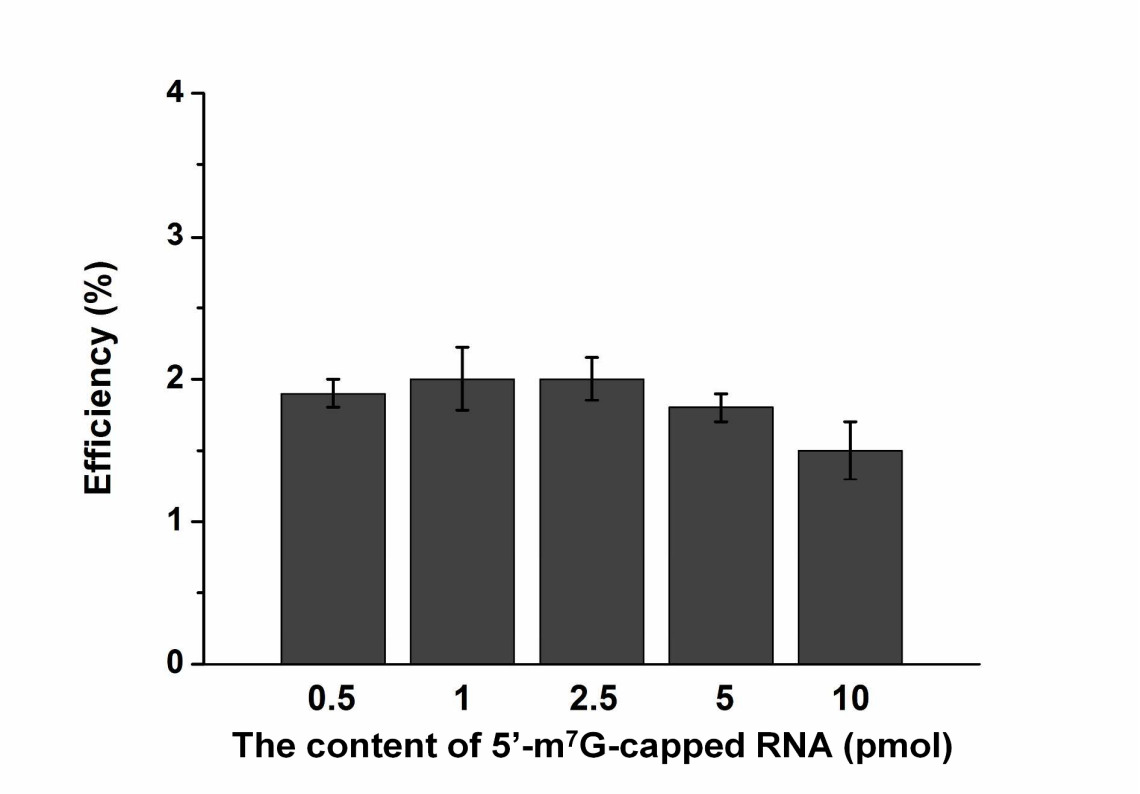


Figure S5. Confirmation of the detected m^7G by high resolution mass spectrometry analysis. (A) Product ion spectrum of the m^7G standard. (B) Product ion spectrum of the detected m^7G from rice mRNA digested by S1 nuclease. (C) Product ion spectrum of the detected m^7G from rice mRNA digested by phosphodiesterase I.

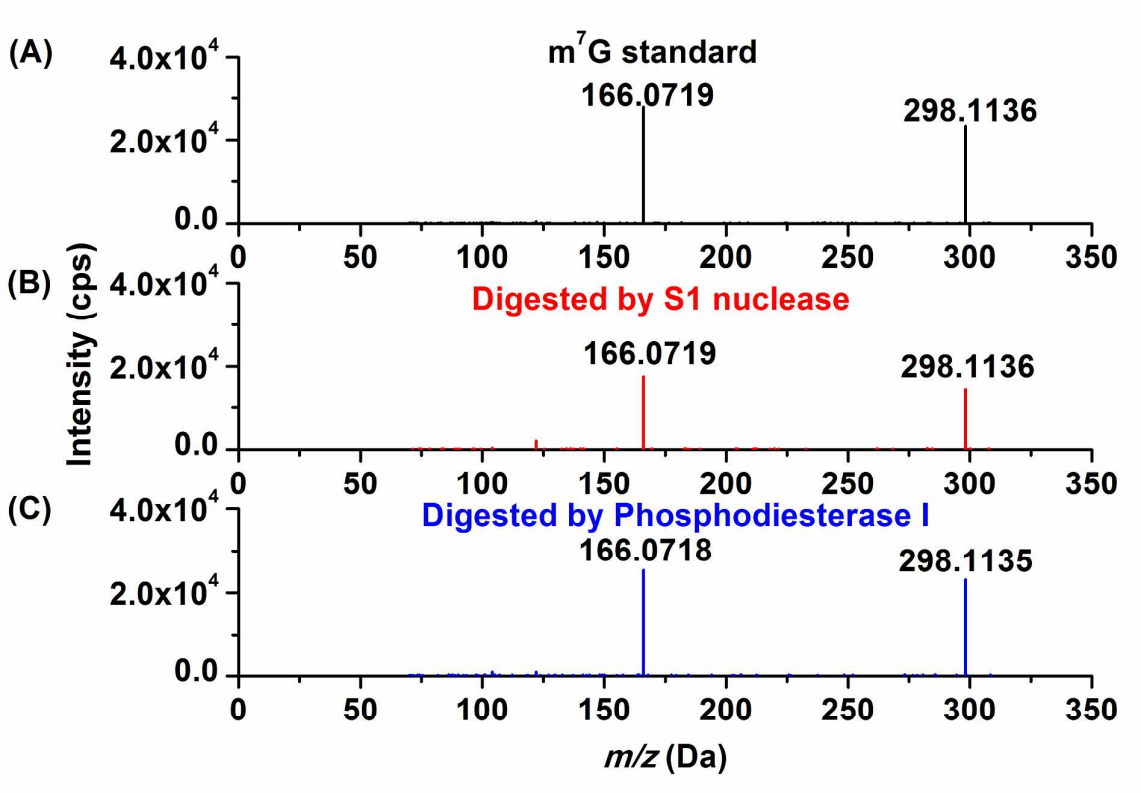
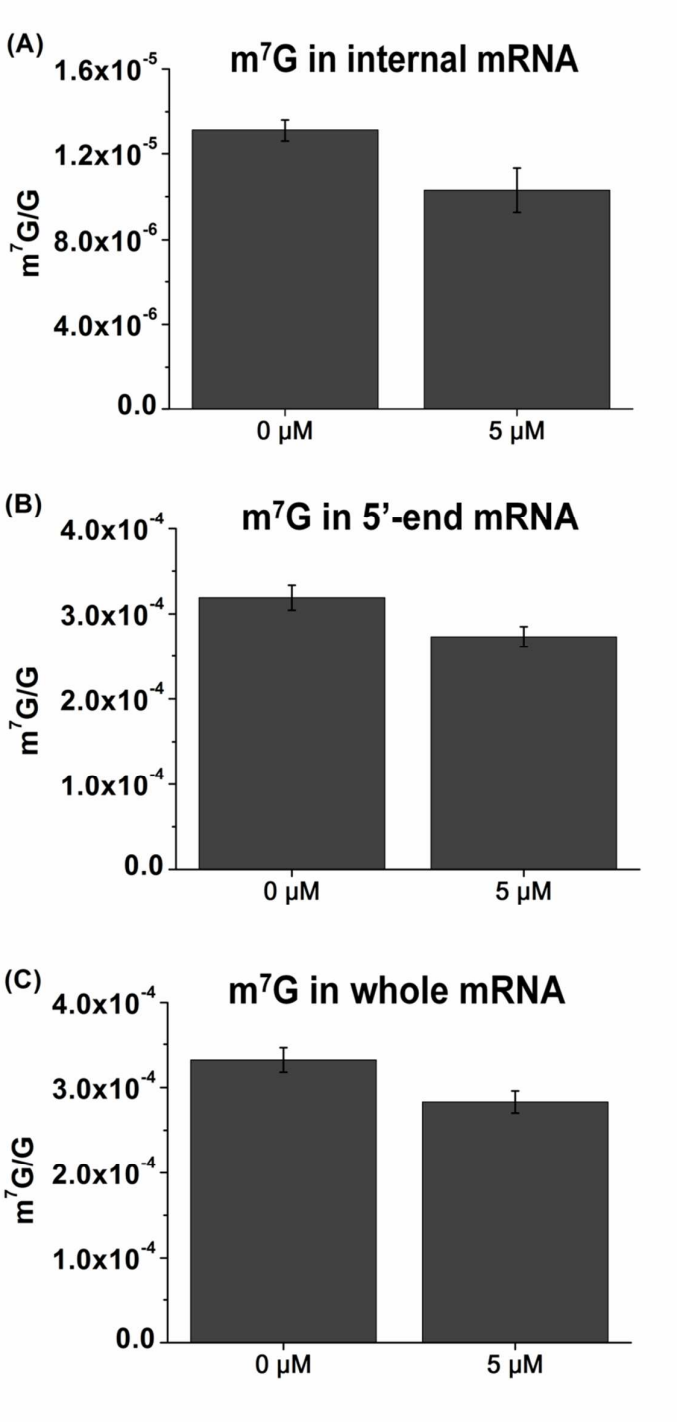


Figure S6. Changes in the levels of internal (A) and 5' cap (B) m^7G in mRNA, and the total levels of m^7G in mRNA (C) of 293T cells after treatment with Cd.



References

1. Tang, Y., Zheng, S. J., Qi, C. B., Feng, Y. Q., and Yuan, B. F. (2015) Sensitive and simultaneous determination of 5-methylcytosine and its oxidation products in genomic DNA by chemical derivatization coupled with liquid chromatography-tandem mass spectrometry analysis, *Anal Chem 87*, 3445-3452.

2. Huang, W., Lan, M. D., Qi, C. B., Zheng, S. J., Wei, S. Z., Yuan, B. F., and Feng, Y. Q. (2016) Formation and determination of the oxidation products of 5-methylcytosine in RNA, *Chem Sci 7*, 5495-5502.

3. Yuan, B. F. (2017) Liquid Chromatography-Mass Spectrometry for Analysis of RNA Adenosine Methylation, *Methods Mol Biol 1562*, 33-42.