Supporting Information

For

Formation and Determination of the Oxidation Products of 5-Methylcytosine in RNA

Wei Huang,[1] Meng-Dan Lan,[1] Chu-Bo Qi,[1,2] Shu-Jian Zheng,[1] Shao-Zhong Wei,[2] Bi-Feng Yuan[1,*] Yu-Qi Feng[1]

[1] Key Laboratory of Analytical Chemistry for Biology and Medicine (Ministry of Education), Department of Chemistry, Wuhan University, Wuhan 430072, P.R. China

[2] Department of Pathology, Hubei Cancer Hospital, Wuhan, Hubei 430079, P.R. China

*To whom correspondence should be addressed. Tel.:+86-27-68755595; fax: +86-27-68755595. E-mail address: bfyuan@whu.edu.cn

The Supporting Information includes following items:

Optimization of Labeling Conditions by BDEPE

We first investigated TEA contents in reaction solution ranging from 0 to 8 mM. Generally, 1 μM 5-mC and 4 mM labeling reagents (molar ratio of labeling reagents/5-mC, 4000/1) were dissolved in ACN; and then, TEA was added followed by incubation at 60ºC for 1 h. The reactions were stopped by immediate freezing at -80ºC. Then, the reaction temperature was optimized in the range from 30 to 70ºC. 4 mM labeling reagents and 4 mM TEA were added to the reaction solution and incubated for 1 h. The concentrations of labeling reagents were also optimized, and the reactions were incubated at 60°C with 4 mM of TEA for 1 h. Finally, the labeling time was optimized.

We optimized the labeling conditions by BDEPE. As for TEA concentration, our results showed that 4 mM TEA was suitable for the reaction (Figure S4A). As for reaction temperature, our results indicated that 60°C was appropriate to obtain good chemical labeling (Figure S4B). In addition, the results showed that 4 mM BDEPE was sufficient for the chemical labeling (Figure S4C). Finally, we optimized the reaction time, and the results demonstrated that 3 h was sufficient for the chemical labeling (Figure S4D). Taken together, the optimized conditions for 5-mC by BDEPE were under 60°C for 3 h with 4 mM BDEPE and TEA (Figure S4).

Optimization of Labeling Conditions by BPPE

We optimized the labeling conditions by BPPE. As for TEA concentration, the largest peak area can be achieved when 2 mM TEA was added. As for reaction temperature, our results indicated that 60°C was appropriate to obtain good chemical labeling. In addition, the results showed that 16 mM BPPE was sufficient for the chemical labeling. Finally, reaction time was

optimized, and our results demonstrated that 16 h was sufficient for the chemical labeling. Taken together, the optimized conditions for 5-mC by BDEPE were under 60°C for 16 h with 16 mM BPPE and 2 mM TEA (Figure S5).

Optimization of Labeling Conditions by BTA

We optimized the labeling conditions by BTA. As for TEA concentration, the largest peak area can be achieved when 2 mM TEA was added. As for reaction temperature, our results indicated that 60°C was appropriate to obtain good chemical labeling. In addition, the results showed that 6 mM BTA was sufficient for the chemical labeling. Finally, reaction time was optimized, and our results demonstrated that 3 h was sufficient for the chemical labeling. Taken together, the optimized conditions for 5-mC by BTA were under 60°C for 3 h with 6 mM BTA and 2 mM TEA (Figure S6).

Optimization of Labeling Conditions by BPB

We optimized the labeling conditions by BPB. As for TEA concentration, the largest peak area can be achieved without TEA. As for reaction temperature, our results indicated that 60°C was appropriate to obtain good chemical labeling. In addition, the results showed that 6 mM BPB was sufficient for the chemical labeling. Finally, reaction time was optimized, and our results demonstrated that 3 h was sufficient for the chemical labeling. Taken together, the optimized conditions for 5-mC by BPB were under 60°C for 4 h with 6 mM BPB (Figure S7).

Isolation of different RNA species

To extract 28S rRNA and 18S rRNA, total RNA was separated by 1.2% agarose gel with 1×TAE at 170 V for 30 min. The gel was stained by GelRed (Invitrogen) and visualized by a gel documentation system (Jiapeng, Shanghai, China). 28S rRNA and 18S rRNA were

excised from the gel and then extracted using the E.Z.N.A.® Gel Extraction Kit (Omega Bio-Tek Inc., Norcross, GA) (Figure S10A). The purified 28S rRNA and 18S rRNA were confirmed by agarose electrophoresis (Figure S10B).

Small RNA (< 200 nt) was purified from mouse liver tissue using the E.Z.N.A.® MiRNA Kit (Omega Bio-Tek Inc., Norcross, GA) according to the manufacture's recommended procedure. The extracted small RNA was confirmed by 10% denaturing polyacrylamide gel electrophoresis (PAGE) containing 7 M urea with 1×TBE at 150 V for 35 min (Figure S10C).

To extract mRNA from total RNA, two successive polyA$^+$-based selections were conducted using the Promega PolyATtract® mRNA Isolation System (Madison, WI) according to the manufacture's recommended procedure. Since mRNA normally doesn't contain the modified nucleoside of N^6,N^6-dimethyladenosine ($m^6{}_2A$); therefore, the content of $m^6{}_2A$ in purified mRNA is typically used to evaluated the purity of mRNA.[1] The level of $m^6{}_2A$ in purified mRNA was determined by LC-MS/MS. The purified mRNA from each step isolation were named "mRNA P1" and "mRNA P2", respectively (Figure S11). The results showed that purity of mRNA by two successive rounds of purification were 99.67%. Therefore, here we purify mRNA by two successive rounds of purification.

Table S1. The optimized conditions for the chemical labeling reactions.

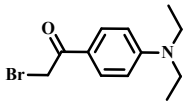
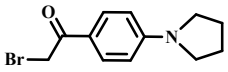
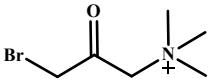
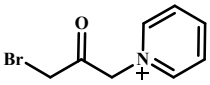
Labeling reagents	Molecular structure	Reaction concentration (mM)	Reaction time (h)	Reaction temperature (ºC)	TEA concentration (mM)
BDEPE		4	3	60	4
BPPE		16	16	60	2
BTA		6	3	60	2
BPB		6	4	60	0

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

Analytes	Precursor ion	Product ion	DP/V	EP / V	CEP / V	CE / V	CXP / V
A	268.4	136.2	15.0	5.0	16.6	23.0	2.0
C	244.4	112.1	20.0	8.0	10.0	21.3	2.4
G	284.5	152.2	40.0	5.0	9.0	23.0	6.0
U	245.4	113.1	24.7	6.0	13.0	13.7	3.8
5-mC	258.0	126.1	28.8	3.4	16.3	19.0	2.8
5-hmC	274.1	124.1	21.0	6.0	13.0	14.0	5.0
5-foC	272.2	140.2	22.0	4.0	14.0	16.0	5.0
5-caC	288.3	156.0	40.0	3.0	11.0	16.8	3.0
5-mC-BDEPE	429.2	297.1	58.0	6.0	16.2	30.0	5.0
5-mC-BPPE	427.2	295.2	50.0	6.7	16.4	29.4	4.8
5-mC-BTA	353.2	294.3	55.6	9.0	12.4	21.8	3.9
5-mC-BPB	373.1	294.1	50.0	6.1	14.3	21.6	4.5
5-hmC-BDEPE	445.2	313.3	52.0	8.0	16.0	25.0	3.3
5-hmC-BPPE	443.2	311.3	52.3	13.4	19.8	22.0	11.2
5-hmC-BTA	369.3	310.4	59.0	7.0	14.8	26.0	3.3
5-hmC-BPB	389.1	310.1	45.6	7.8	15.4	20.7	2.3
5-foC-BDEPE	443.3	311.2	50.0	8.0	17.4	26.5	4.1
5-foC-BPPE	441.2	309.2	48.9	8.7	14.1	30.0	4.7
5-foC-BTA	367.3	308.1	55.0	8.0	13.1	28.2	4.7
5-foC-BPB	387.2	308.1	36.0	8.7	15.1	19.8	2.5
5-caC-BDEPE	648.4	516.3	56.0	6.0	18.0	26.2	5.2
5-caC-BPPE	475.3	343.3	18.0	3.6	23.5	30.0	5.0
5-caC-BTA	248.6	154.2	51.0	9.0	20.6	33.8	2.8
5-caC-BPB	268.4	189.4	53.0	6.1	11.2	25.0	1.9

Table S3. Labeling efficiencies of 5-mC, 5-hmC, 5-foC and 5-caC by different labeling reagents.

Analytes	Labeling efficiency (%)			
	BDEPE	BPPE	BTA	BPB
5-mC	99.8	56.7	65.2	50.4
5-hmC	99.5	55.2	62.1	28.2
5-foC	99.2	50.1	50.3	20.3
5-caC	99.5	75.0	72.1	25.1

Table S4. Calibration curves for the analysis of 5-mC, 5-hmC, 5-foC and 5-caC by BDEPE labeling coupled with LC-ESI-MS/MS analysis.

Compound molar ratio	Linear range	Calibration curve data		
		Slope	Intercept	R^2
5-mC/10^3 G	0.2-50	0.0993	0.0077	0.9996
5-hmC/10^6 G	0.2-100	0.0009	0.00015	0.9995
5-foC/10^6 G	0.2-50	0.0004	0.00011	0.9937
5-caC/10^7 G	0.2-50	0.00005	0.00003	0.9916

Table S5. Accuracy and precision for the determination of 5-mC by BDEPE labeling coupled with LC-ESI-MS/MS analysis.

	Nominal 5-mC/10^3 G	0.20	0.50	1.00	2.00	5.00	10.00	20.00	50.00
Day 1 *n*=3	Measured mean 5-mC/10^3 G	0.18	0.55	1.08	1.93	5.32	9.18	21.45	54.10
	RSD (%)	15.0	14.8	2.2	3.5	8.2	7.7	1.6	7.2
	RE (%)	10.2	10.1	7.8	-3.5	6.4	-8.2	7.3	8.2
Day 2 *n*=3	Measured mean 5-mC/10^3 G	0.17	0.53	0.89	1.86	5.55	9.36	20.70	53.31
	RSD (%)	11.2	8.2	8.8	13.6	4.8	6.3	9.5	12.0
	RE (%)	14.9	5.9	-11.3	-7.2	11.0	-6.4	3.5	6.6
Day 3 *n*=3	Measured mean 5-mC/10^3 G	0.21	0.57	0.93	1.79	5.37	9.49	20.82	51.01
	RSD (%)	9.3	9.7	7.3	8.1	7.5	1.1	1.7	11.5
	RE (%)	5.1	14.0	-7.2	-10.1	7.4	-5.1	4.1	2.0

Table S6. Accuracy and precision for the determination of 5-hmC by BDEPE labeling coupled with LC-ESI-MS/MS analysis.

	Nominal 5-hmC/10^6 G	0.20	0.50	1.00	2.00	5.00	10.00	20.00	50.00	100.00
Day 1 *n*=3	Measured mean 5-hmC/10^6 G	0.23	0.54	1.12	1.85	5.27	9.28	21.22	50.12	102.15
	RSD (%)	11.0	10.9	4.2	3.5	5.2	7.7	8.2	2.1	9.5
	RE (%)	15.0	8.4	12.1	-7.5	5.4	-7.2	6.1	0.2	2.2
Day 2 *n*=3	Measured mean 5-hmC/10^6 G	0.19	0.53	0.90	1.76	5.52	9.46	20.36	51.48	94.63
	RSD (%)	13.0	8.2	8.8	13.6	9.8	10.3	9.5	7.3	8.9
	RE (%)	-5.1	6.1	-11.3	-12.0	10.4	-5.4	1.8	3.0	-5.4
Day 3 *n*=3	Measured mean 5-hmC/10^6 G	0.18	0.57	0.95	1.88	5.37	9.39	20.32	52.01	108.37
	RSD (%)	15.1	10.7	7.3	8.1	9.1	5.1	7.7	11.6	6.7
	RE (%)	10.1	14.1	-5.2	-6.3	7.4	-6.1	1.6	4.0	8.4

Table S7. Accuracy and precision for the determination of 5-foC by BDEPE labeling coupled with LC-ESI-MS/MS analysis.

	Nominal 5-foC/10^6 G	0.20	0.50	1.00	2.00	5.00	10.00	20.00	50.00
Day 1 *n*=3	Measured mean 5-foC/10^6 G	0.18	0.54	1.05	1.86	5.12	9.47	22.04	51.62
	RSD (%)	15.2	14.9	2.2	1.5	3.2	7.9	3.2	1.7
	RE (%)	10.1	8.8	5.2	-7.1	2.4	-5.3	10.2	3.2
Day 2 *n*=3	Measured mean 5-foC/10^6 G	0.19	0.55	0.89	1.76	5.54	9.36	20.50	52.56
	RSD (%)	14.7	8.2	8.8	13.6	4.8	10.3	9.5	4.5
	RE (%)	5.1	9.9	-11.1	-12.1	10.8	-6.4	2.5	5.1
Day 3 *n*=3	Measured mean 5-foC/10^6 G	0.23	0.58	0.95	1.79	5.35	9.59	20.42	54.80
	RSD (%)	10.2	9.7	7.3	2.1	7.1	1.1	0.7	6.7
	RE (%)	14.9	16.0	-5.0	-10.6	7.0	-4.1	2.1	9.6

Table S8. Accuracy and precision for the determination of 5-caC by BDEPE labeling coupled with LC-ESI-MS/MS analysis.

	Nominal 5-caC/10^7 G	0.20	0.50	1.00	2.00	5.00	10.00	20.00	50.00
Day 1 *n*=3	Measured mean 5-caC/10^7 G	0.22	0.53	1.08	1.93	5.12	10.17	21.52	52.50
	RSD (%)	15.1	14.9	2.2	1.6	5.2	7.8	1.4	2.4
	RE (%)	11.1	6.1	8.2	-3.5	2.4	1.7	7.6	5.0
Day 2 *n*=3	Measured mean 5-caC/10^7 G	0.23	0.51	0.89	1.84	5.41	9.66	20.60	54.30
	RSD (%)	13.5	8.2	8.5	5.6	4.9	10.3	9.5	3.9
	RE (%)	15.0	2.0	-11.0	-8.0	8.2	-4.4	3.0	8.6
Day 3 *n*=3	Measured mean 5-caC/10^7 G	0.18	0.52	0.91	1.89	5.45	9.59	20.35	51.20
	RSD (%)	12.6	9.7	7.3	2.1	7.1	1.1	0.7	6.1
	RE (%)	-10.1	4.2	-9.2	-5.5	9.0	-4.1	1.8	2.4

Table S9. The measured contents of 5-mC, 5-hmC, 5-foC and 5-caC in total RNA from human CRC tissues and tumor adjacent normal tissues.

Number	Tissue	5-mC (/10^3 G)	5-hmC (/10^6 G)	5-foC (/10^6 G)	5-caC (/10^7 G)
01	Tumor	3.6 ± 0.4	5.8 ± 0.4	5.8 ± 0.5	2.6 ± 0.4
	Adjacent	21.9 ± 2.0	93.6 ± 13.0	41.9 ± 7.5	10.9 ± 1.8
02	Tumor	7.4 ± 0.7	12.3 ± 2.0	5.5 ± 0.8	4.0 ± 0.1
	Adjacent	6.9 ± 0.7	16.6 ± 1.6	1.6 ± 0.2	1.6 ± 0.2
03	Tumor	12.2 ± 1.3	6.7 ± 0.9	4.1 ± 0.7	1.7 ± 0.0
	Adjacent	11.9 ± 0.4	56.7 ± 0.1	2.8 ± 0.3	14.3 ± 0.4
04	Tumor	2.0 ± 0.2	10.6 ± 0.3	6.1 ± 0.1	2.4 ± 0.4
	Adjacent	2.6 ± 0.3	18.2 ± 2.0	11.8 ± 0.5	0.4 ± 0.1
05	Tumor	12.8 ± 0.5	17.6 ± 0.9	8.0 ± 0.8	11.0 ± 0.1
	Adjacent	17.7 ± 0.2	25.5 ± 1.3	9.3 ± 1.7	5.0 ± 0.5
06	Tumor	6.0 ± 0.3	23.7 ± 2.9	6.4 ± 0.9	1.3 ± 0.1
	Adjacent	9.5 ± 0.5	29.8 ± 2.7	6.9 ± 0.8	2.9 ± 0.4
07	Tumor	8.2 ± 0.2	16.5 ± 1.1	15.0 ± 1.2	7.1 ± 1.0
	Adjacent	5.9 ± 0.0	31.8 ± 0.6	6.2 ± 1.0	5.6 ± 1.0
08	Tumor	1.7 ± 0.1	5.3 ± 0.5	3.6 ± 0.6	1.1 ± 0.2
	Adjacent	3.5 ± 0.1	6.0 ± 0.8	5.5 ± 1.0	2.0 ± 0.1
09	Tumor	5.5 ± 0.3	4.0 ± 0.6	1.2 ± 0.0	6.1 ± 0.1
	Adjacent	1.6 ± 0.3	16.2 ± 1.2	5.4 ± 1.0	5.8 ± 0.1
10	Tumor	3.8 ± 0.1	6.1 ± 0.3	3.5 ± 0.4	2.7 ± 0.3
	Adjacent	5.2 ± 0.5	21.7 ± 1.9	15.0 ± 1.5	10.3 ± 0.3
11	Tumor	3.3 ± 0.3	6.1 ± 0.3	16.0 ± 0.1	5.5 ± 0.2
	Adjacent	6.9 ± 1.4	16.2 ± 1.5	12.1 ± 1.3	10.8 ± 0.5
12	Tumor	6.3 ± 1.0	2.1 ± 0.3	3.2 ± 0.2	3.9 ± 0.5
	Adjacent	7.6 ± 1.1	3.4 ± 0.6	2.6 ± 0.5	3.1 ± 0.6
13	Tumor	5.0 ± 0.0	31.8 ± 0.9	9.2 ± 0.7	19.3 ± 0.3
	Adjacent	3.0 ± 0.2	52.0 ± 6.3	18.1 ± 3.1	7.2 ± 0.2
14	Tumor	5.6 ± 0.1	17.2 ± 2.2	1.9 ± 0.1	1.0 ± 0.1
	Adjacent	5.4 ± 0.1	25.0 ± 3.3	3.1 ± 0.2	0.8 ± 0.1
15	Tumor	1.9 ± 0.1	12.7 ± 0.6	1.6 ± 0.2	5.8 ± 0.4
	Adjacent	3.0 ± 0.1	45.1 ± 5.7	23.8 ± 3.6	20.3 ± 2.0
16	Tumor	1.7 ± 0.1	37.1 ± 5.1	2.6 ± 0.3	9.2 ± 1.5
	Adjacent	6.7 ± 0.1	73.9 ± 10.4	6.8 ± 1.3	7.3 ± 1.1
17	Tumor	2.6 ± 0.2	24.2 ± 0.9	28.4 ± 1.3	11.9 ± 1.7
	Adjacent	1.1 ± 0.0	41.3 ± 0.3	13.4 ± 1.5	1.2 ± 0.2
18	Tumor	1.4 ± 0.0	35.6 ± 4.3	42.3 ± 3.9	16.2 ± 2.2
	Adjacent	2.5 ± 0.1	72.2 ± 7.9	28.9 ± 0.4	16.5 ± 1.6
19	Tumor	2.6 ± 0.3	41.8 ± 2.2	14.8 ± 0.1	13.7 ± 0.6
	Adjacent	2.1 ± 0.0	65.3 ± 2.2	38.4 ± 6.2	12.7 ± 1.4
20	Tumor	1.8 ± 0.1	39.9 ± 4.4	15.8 ± 1.5	13.5 ± 0.7
	Adjacent	1.2 ± 0.1	52.6 ± 4.4	19.5 ± 1.3	6.8 ± 1.0

Table S10. The measured contents of 5-mC, 5-hmC, 5-foC and 5-caC in total RNA from human HCC tissues and tumor adjacent normal tissues.

Number	Tissue	5-mC (/10^3 G)	5-hmC (/10^6 G)	5-foC (/10^6 G)	5-caC (/10^7 G)
01	Tumor	2.8 ± 0.2	7.3 ± 0.3	3.0 ± 0.4	4.3 ± 0.6
	Adjacent	1.9 ± 0.0	11.2 ± 0.5	2.1 ± 0.1	7.8 ± 0.0
02	Tumor	2.0 ± 0.1	7.2 ± 0.1	2.2 ± 0.3	1.6 ± 0.2
	Adjacent	2.2 ± 0.0	8.3 ± 0.4	1.6 ± 0.2	4.7 ± 0.2
03	Tumor	2.2 ± 0.0	7.9 ± 0.6	1.3 ± 0.1	1.1 ± 0.1
	Adjacent	2.6 ± 0.0	9.9 ± 0.2	2.0 ± 0.1	1.5 ± 0.0
04	Tumor	1.8 ± 0.0	4.9 ± 0.1	1.5 ± 0.2	1.4 ± 0.1
	Adjacent	3.4 ± 0.1	14.8 ± 0.1	1.8 ± 0.0	0.9 ± 0.0
05	Tumor	7.0 ± 0.2	5.4 ± 0.5	17.1 ± 0.7	4.0 ± 0.2
	Adjacent	3.3 ± 0.1	9.7 ± 0.4	4.0 ± 0.7	11.2 ± 1.5
06	Tumor	4.6 ± 0.0	14.3 ± 1.2	8.6 ± 0.5	13.0 ± 0.8
	Adjacent	4.3 ± 0.2	23.3 ± 1.6	5.4 ± 0.5	4.2 ± 0.4
07	Tumor	6.7 ± 0.0	6.5 ± 0.7	10.2 ± 1.0	3.9 ± 0.3
	Adjacent	5.9 ± 0.2	9.3 ± 0.1	7.8 ± 0.5	5.2 ± 0.6
08	Tumor	8.6 ± 0.2	10.0 ± 0.6	10.5 ± 1.6	4.5 ± 0.3
	Adjacent	6.1 ± 0.2	21.1 ± 1.1	9.1 ± 0.1	4.0 ± 0.2

Table S11. The measured contents of 5-mC, 5-hmC, 5-foC and 5-caC in mRNA from human HCC tissues and tumor adjacent normal tissues.

Number	Tissue	5-mC (/10^3 G)	5-hmC (/10^4 G)	5-foC (/10^4 G)	5-caC (/10^6 G)
01	Tumor	4.1 ± 0.7	2.5 ± 0.1	1.1 ± 0.2	7.8 ± 1.0
	Adjacent	2.6 ± 0.3	4.0 ± 0.0	1.0 ± 0.1	14.8 ± 2.2
02	Tumor	1.7 ± 0.0	2.4 ± 0.2	0.8 ± 0.1	3.1 ± 0.4
	Adjacent	2.0 ± 0.0	3.0 ± 0.2	0.7 ± 0.1	8.1 ± 0.6
03	Tumor	2.5 ± 0.2	1.6 ± 0.2	4.7 ± 0.1	n.q.*
	Adjacent	10.6 ± 0.0	5.7 ± 0.2	1.7 ± 0.1	n.q.
04	Tumor	7.1 ± 0.6	2.8 ± 0.4	0.7 ± 0.0	n.q.
	Adjacent	12.4 ± 0.2	4.7 ± 0.4	4.1 ± 0.3	n.q.
05	Tumor	3.0 ± 0.1	2.2 ± 0.2	4.2 ± 0.3	12.5 ± 0.6
	Adjacent	3.5 ± 0.0	5.4 ± 0.3	1.8 ± 0.1	21.4 ± 0.2
06	Tumor	4.1 ± 0.4	4.2 ± 0.2	4.3 ± 0.0	23.8 ± 3.5
	Adjacent	2.4 ± 0.1	8.2 ± 0.8	2.4 ± 0.0	6.8 ± 0.7
07	Tumor	9.5 ± 0.0	3.3 ± 0.4	4.4 ± 0.3	17.1 ± 0.5
	Adjacent	1.7 ± 0.1	4.5 ± 0.2	2.6 ± 0.2	8.5 ± 1.0
08	Tumor	10.6 ± 0.6	4.7 ± 0.7	3.9 ± 0.5	13.8 ± 1.5
	Adjacent	8.6 ± 0.4	17.0 ± 0.3	6.7 ± 1.1	26.2 ± 1.8

*Not quantified.

Figure S1. Product ions spectra of BPPE labeled 5-mC (A), 5-hmC (B), 5-foC (C), and 5-caC (D).

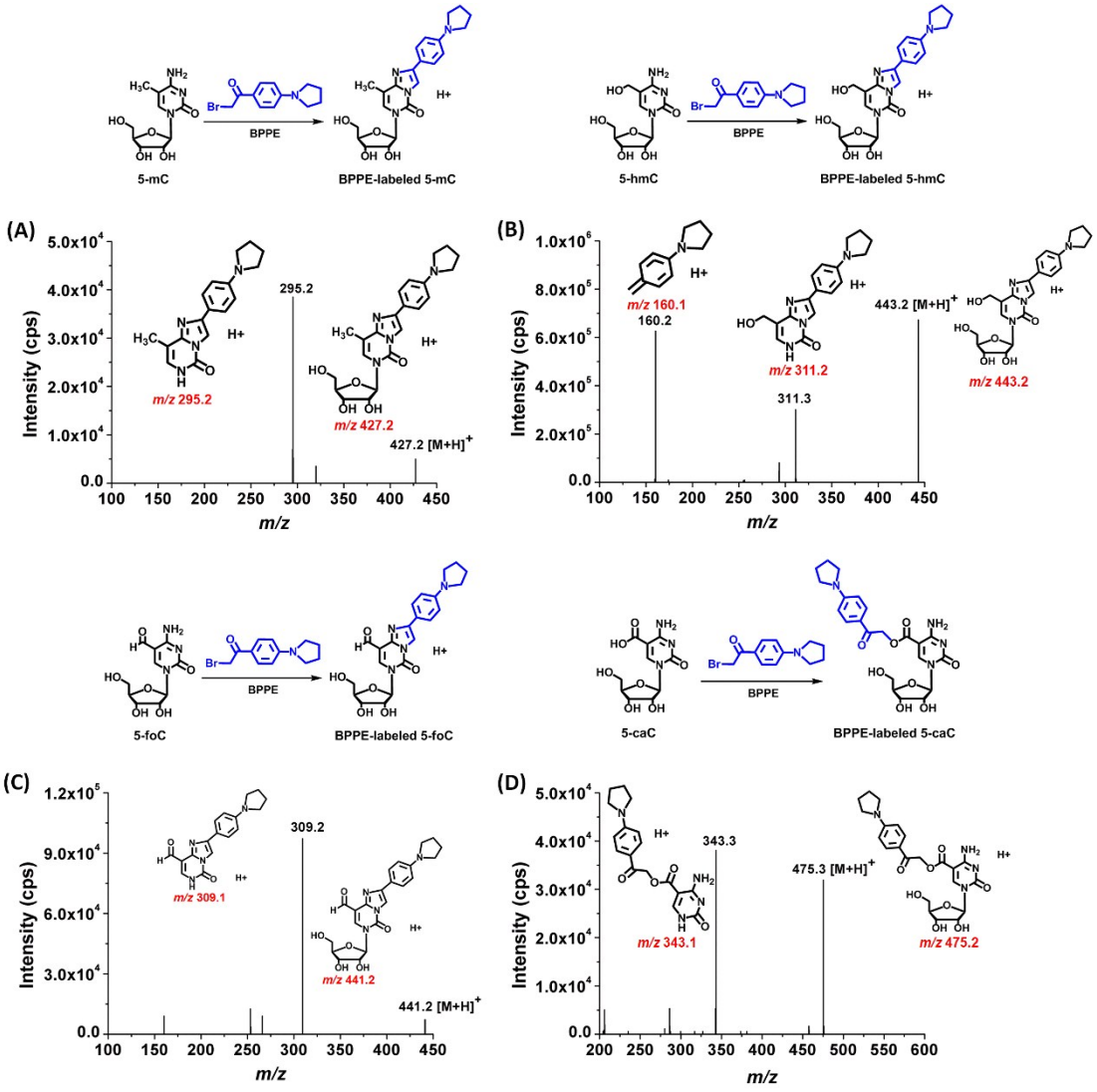


Figure S2. Product ions spectra of BTA labeled 5-mC (A), 5-hmC (B), 5-foC (C), and 5-caC (D).

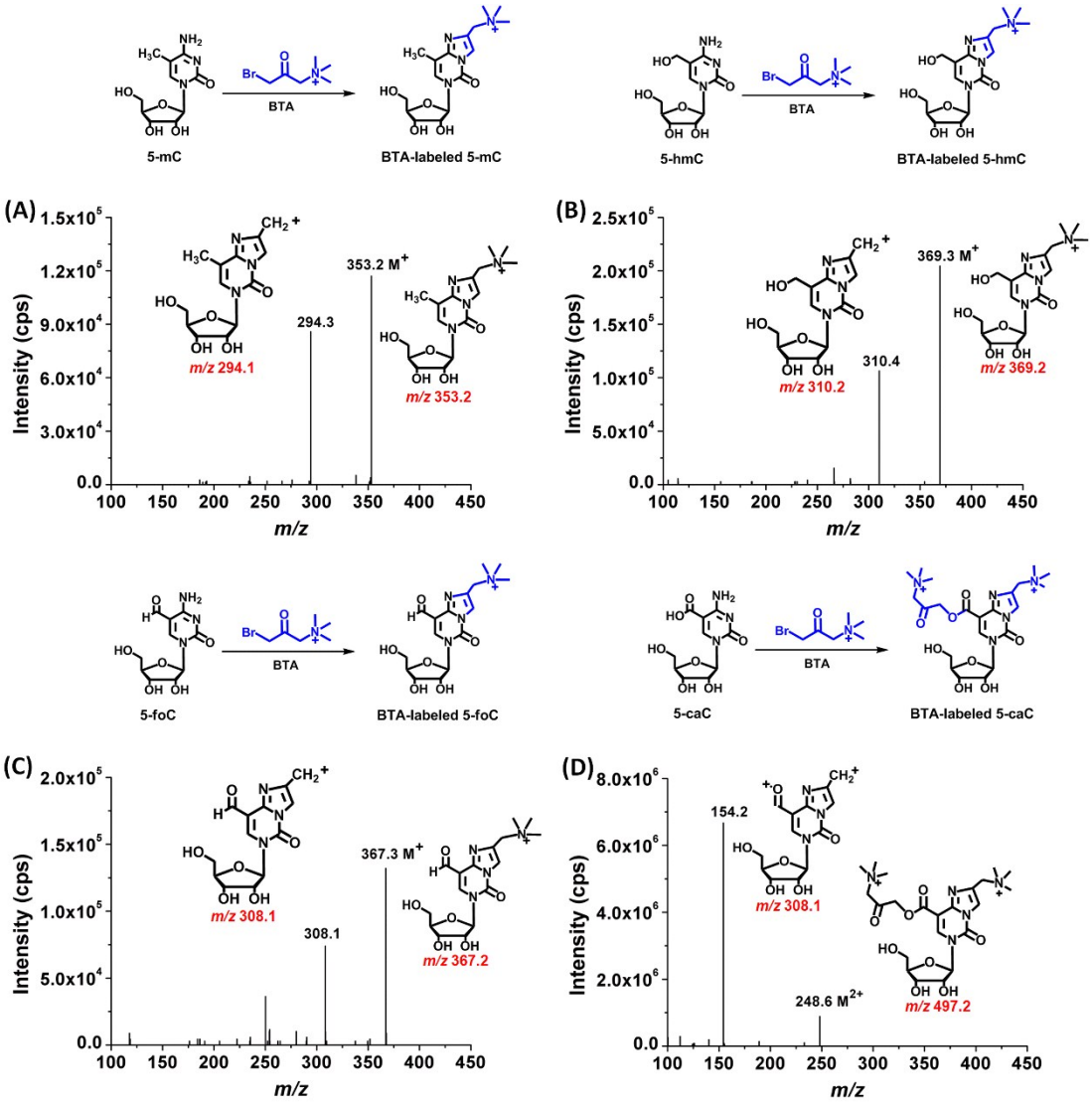


Figure S3. Product ions spectra of BPB labeled 5-mC (A), 5-hmC (B), 5-foC (C), and 5-caC (D).

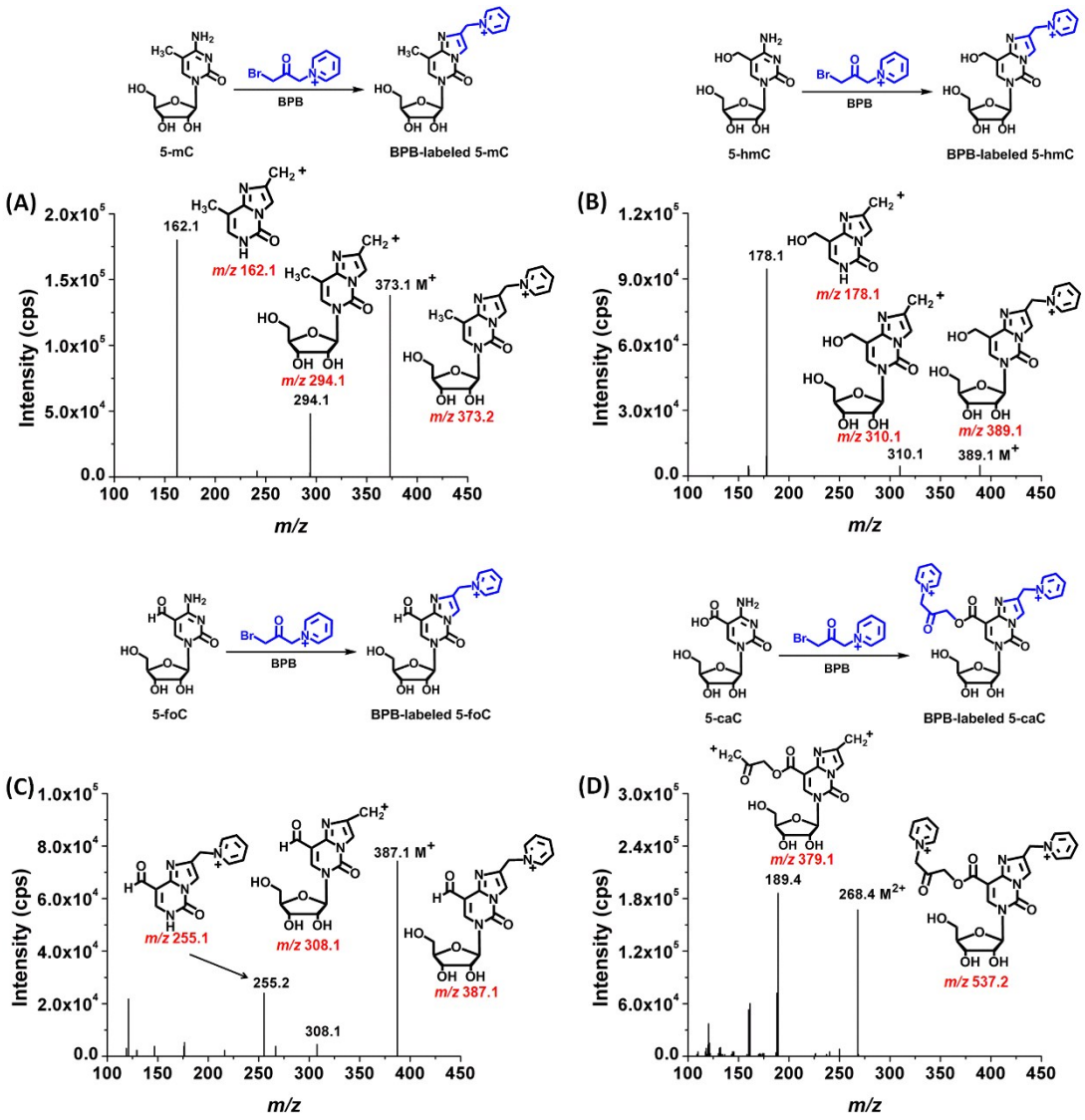


Figure S4. Optimization of labeling conditions for 5-mC by BDEPE. The effects of (A) TEA concentration, (B) reaction temperature, (C) BDEPE concentration, and (D) reaction time on the labeling of 5-mC.

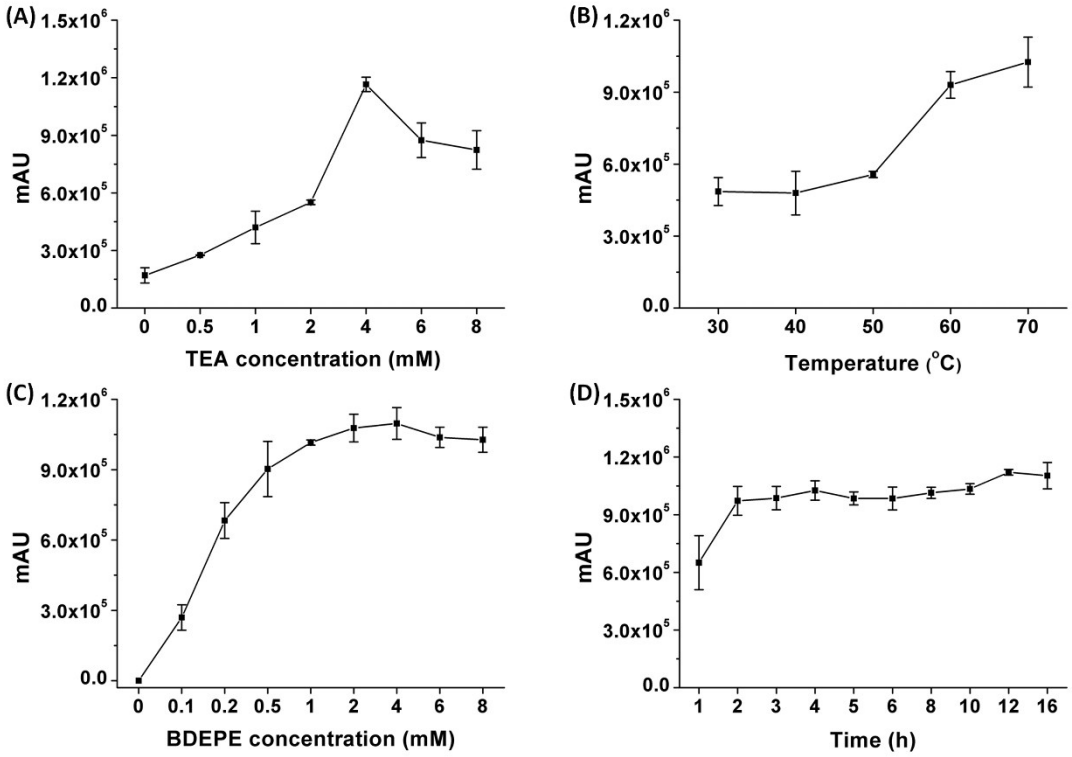


Figure S5. Optimization of labeling conditions for 5-mC by BPPE. The effects of (A) TEA concentration, (B) reaction temperature, (C) BPPE concentration, and (D) reaction time on the labeling of 5-mC.

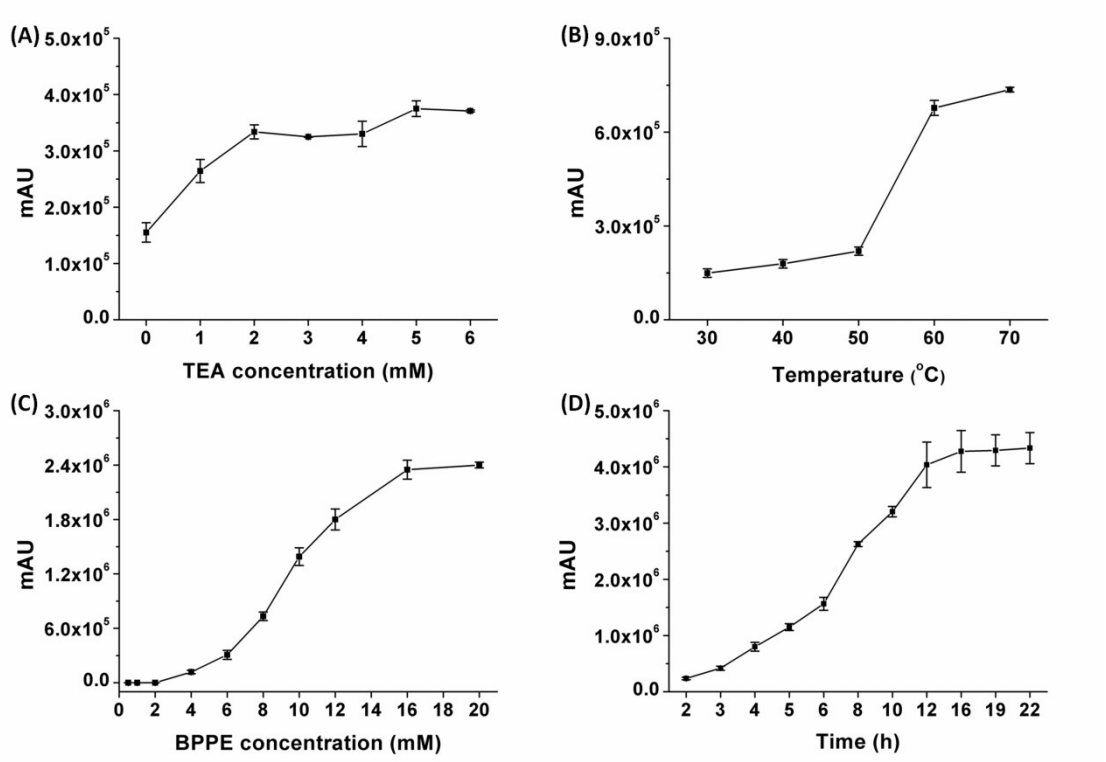


Figure S6. Optimization of labeling conditions for 5-mC by BTA. The effects of (A) TEA concentration, (B) reaction temperature, (C) BTA concentration, and (D) reaction time on the labeling of 5-mC.

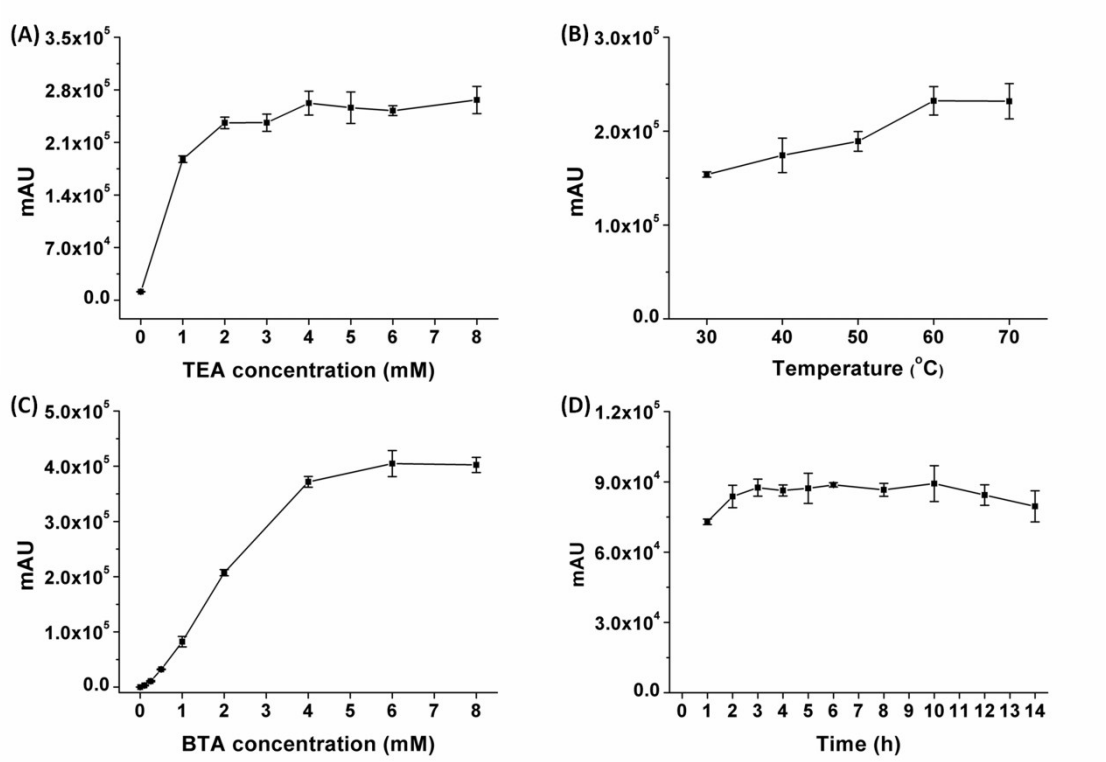


Figure S7. Optimization of labeling conditions for 5-mC by BPB. The effects of (A) TEA concentration, (B) reaction temperature, (C) BPB concentration, and (D) reaction time on the labeling of 5-mC.

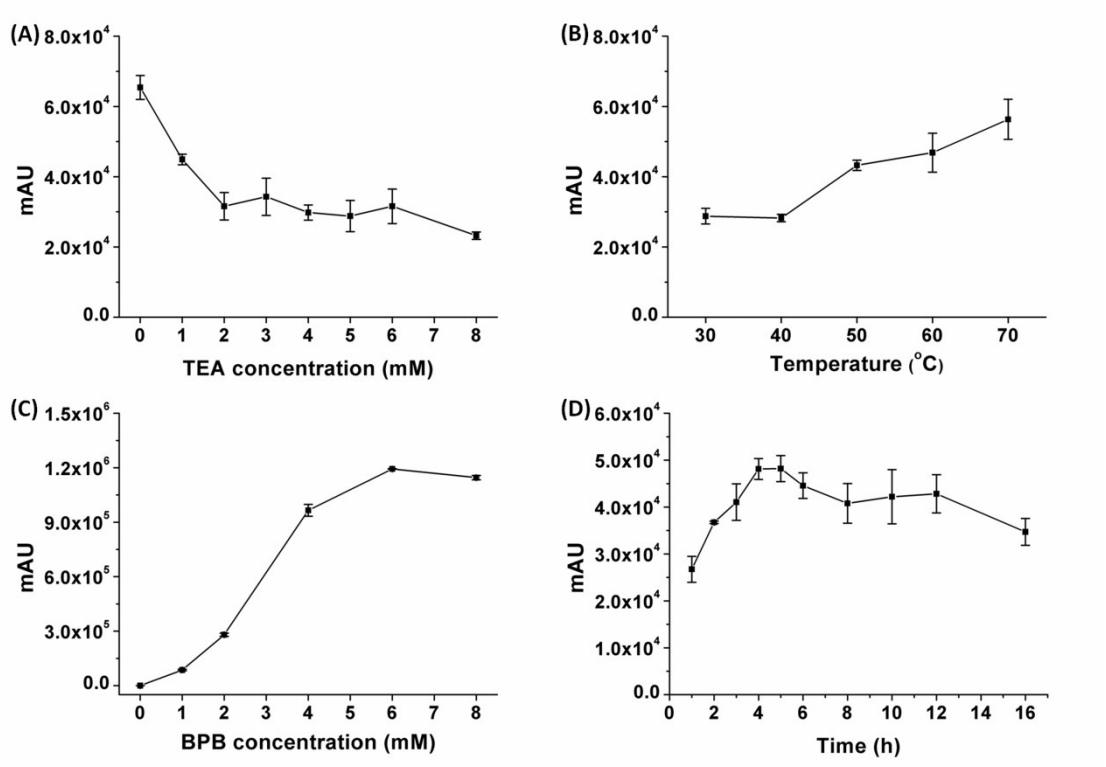


Figure S8. Extracted ion chromatograms of 5-mC, 5-hmC, 5-foC, and 5-caC after labeling by (A) BPPE, (B) BTA, and (C) BPB under optimized conditions. The amount of 5-mC, 5-hmC, 5-foC, and 5-caC were 200 fmol, respectively.

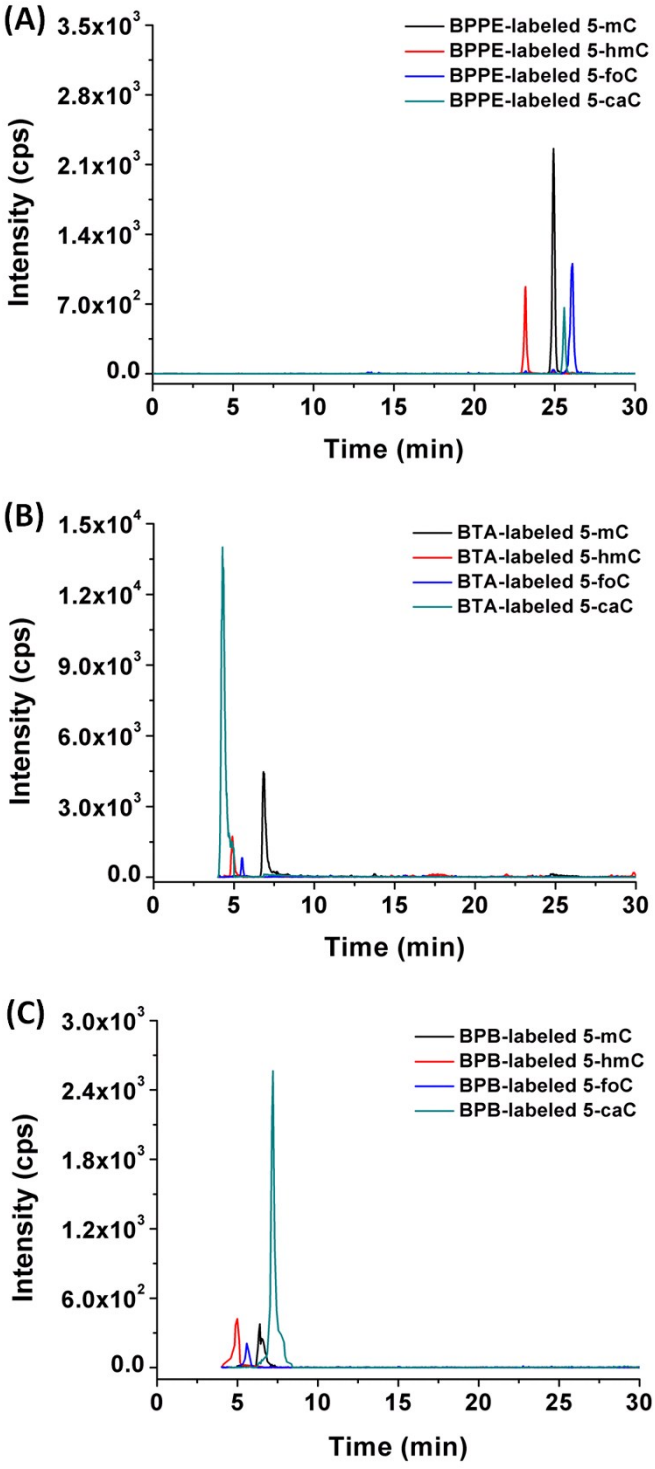


Figure S9. The retention of the labeling reagents under the analytical conditions of LC-ESI-MS/MS.

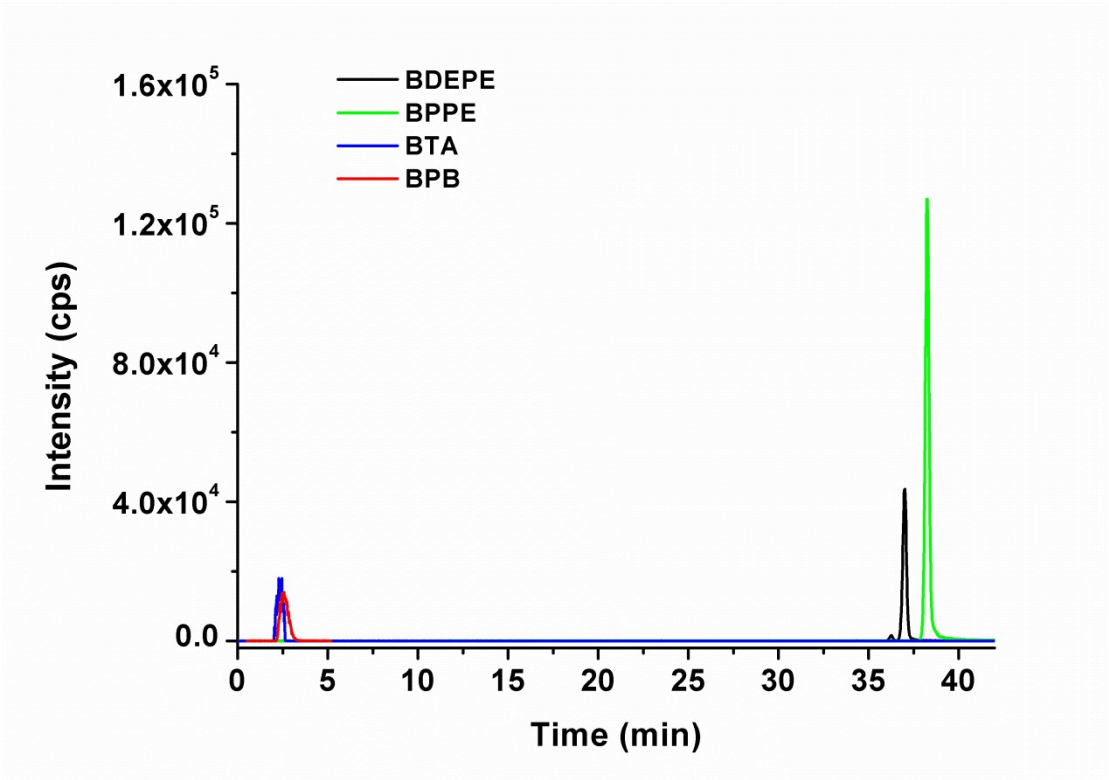


Figure S10. Isolation of 28S rRNA, 18S rRNA, and small RNA (< 200 nt). (A) Separation of total RNA by agarose gel electrophoresis. Lane 1, RNA ladder; lane 2, total RNA of mouse liver tissue. (B) Examination of the purified 28S rRNA and 18S rRNA by agarose gel electrophoresis. Lane 1, RNA ladder 6000; lane 2, purified 28S rRNA; lane 3, purified 18S rRNA. (C) Polyacrylamide gel electrophoresis of the extracted small RNA. Lane 1, RNA ladder; lane 2, purified small RNA (< 200 nt).

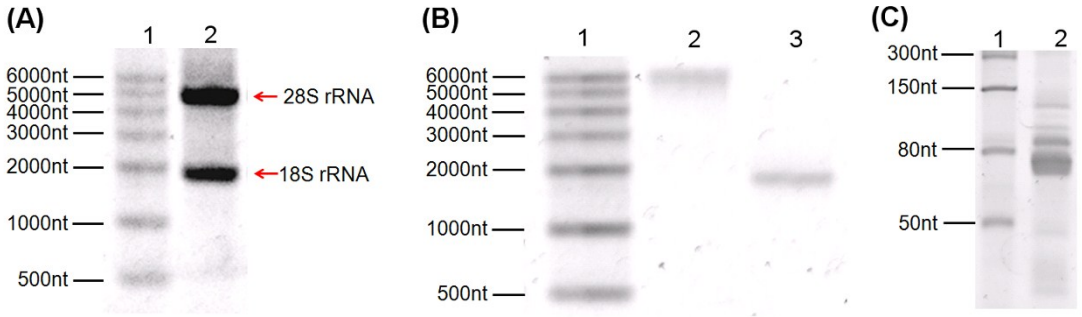
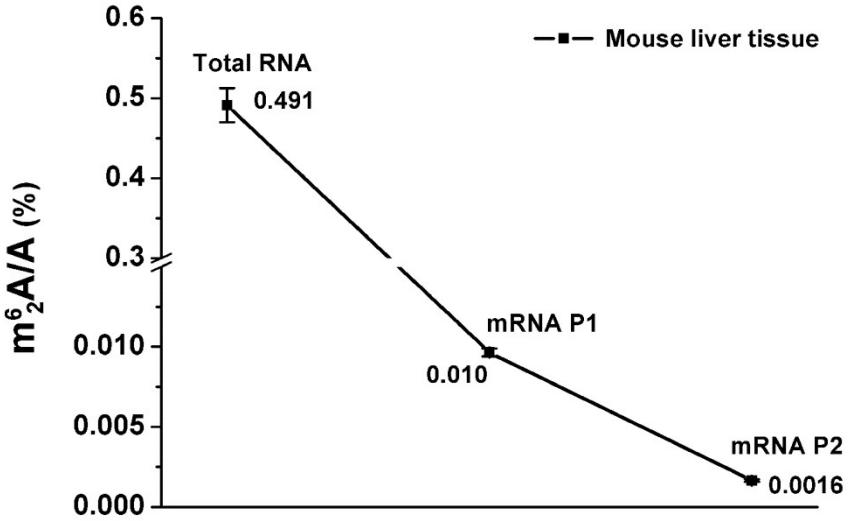


Figure S11. Evaluation of the purity of the isolated mRNA. mRNA normally doesn't contain N^6,N^6-dimethyladenosine (m^6_2A); therefore, the content of m^6_2A in purified mRNA is typically used to evaluate the purity of mRNA. The values of the y axis represent m^6_2A level in RNA samples. In total RNA, the content of m^6_2A is 0.491% (m^6_2A/A); after purification by mRNA isolation kit once, the content of m^6_2A decreased to 0.010% (m^6_2A/A); after two successive rounds of purification by mRNA isolation kit, the content of m^6_2A decreased to 0.0016% (m^6_2A/A). The calculated purity of mRNA by one-time purification and two successive rounds of purification were 97.96% and 99.67%, respectively. Therefore, here we purify mRNA by two successive rounds of purification.



References.

1. X. Li, P. Zhu, S. Ma, J. Song, J. Bai, F. Sun and C. Yi, *Nat Chem Biol*, 2015, 11, 592-597.