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Diazo reagent labeling with mass spectrometry analysis for sensitive determination of ribonucleotides in living organisms

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ABSTRACT

Ribonucleotides analogues and their related phosphorylated metabolites play critical roles in tumor metabolism. However, determination of the endogenous ribonucleotides from complex biological matrix is still challenging due to their high structural similarity and high polarity that will lead to the low retention and low detection sensitivities by liquid chromatogram mass spectrometry analysis. In this study, we developed the diazo reagent labeling strategy with mass spectrometry analysis for sensitive determination of ribonucleotides in living organism. A pair of light and heavy stable isotope labeling reagents, 2-(diazomethyl)-*N*-methyl-*N*-phenyl-benzamide (2-DMBA) and d_5-2-(diazomethyl)-*N*-methyl-*N*-phenyl-benzamide (d_5-2-DMBA), were synthesized to label ribonucleotides. 2-DMBA showed high specificity and high efficiency for the labeling of ribonucleotides. Our results demonstrated that the detection sensitivities of 12 ribonucleotides increased by 17-174 folds upon 2-DMBA labeling. The obtained limits of detection (LODs) of ribonucleotides ranged from 0.07 fmol to 0.41 fmol. Using this method, we achieved the sensitive and accurate detection of ribonucleotides from only a few cells (8 cells). To the best of our knowledge, this is the highest detection sensitivities for ribonucleotides ever reported. In addition, we found that the contents of almost all of these ribonucleotides were significantly increased in human breast carcinoma tissues compared to tumor-adjacent normal tissues, suggesting that endogenous ribonucleotides may play certain functional roles in the regulation of cancer development and formation. This method also can be potentially applied in the analysis of phosphorylated compounds.

Keywords: 2-(diazomethyl)-*N*-methyl-*N*-phenyl-benzamide; ribonucleotide; mass spectrometry; chemical labeling.

INTRODUCTION

Ribonucleotides are important biomolecules and consist of nitrogenous base, sugar, and phosphate group. Apart from being the building blocks for RNA molecules, ribonucleotides are involved in many important cellular processes, including cellular signal transduction and enzyme regulation and metabolism.[1] Ribonucleotides analogues are usually used as anticancer drugs, and their related phosphorylated metabolites play critical roles in tumor cell metabolism.[2] Thus, profiling the levels of ribonucleotides could provide information for understanding the relationships between ribonucleotides and diseases, and develop novel means for treatment of diseases.

Some methods have been established to detect cellular ribonucleotides. Capillary electrophoresis (CE) with ultraviolet (UV) detection, high-performance liquid chromatography (HPLC) with UV detection and thin-layer chromatography analysis were applied to detect ribonucleotides.[3-6] But these methods generally have low detection sensitivities and weak capability on identification for ribonucleotides. Liquid chromatography mass spectrometry (LC-MS) has been frequently employed for analysis of ribonucleotides.[7-10] Ribonucleotides have weak retention on the reversed-phase column due to their high priority, which could lead to the poor separation of ribonucleotides. To improve the retention of ribonucleotides on reversed-phase chromatographic separation, ion-pairing reagents were usually added to mobile phases.[11,12] But these additive reagents could contaminate mass spectrometer and induce ion suppression to analytes, which eventually cause the low detection sensitivities of analytes. Hydrophilic interaction chromatography (HILIC) coupled with MS is an alternative technique for detection of ribonucleotides, but peak tailing and drifting are frequently observed for

separation of ribonucleotides, which would lead to the poor reproducibility as well as low detection sensitivities.[13]

In recent years, chemical labeling strategy has gained great attention for improving the detection performance of analytes during mass spectrometry analysis.[14-19] Chemical labeling strategy intends to add a specific group to the target analytes and endow the desired properties of the labeled products, which therefore can be utilized to enhance the detection sensitivity or improve the chromatographic separation of target analytes. We and others recently developed the chemical strategy for analysis of methylated nucleotides.[20,21] But ribonucleotides can easily lose the phosphate group and generally are not very stable, especially for the nucleoside triphosphates.[22] Thus, the ideal chemical labeling reaction for ribonucleotides should be finished in mild conditions and in relatively short time. In addition, accurate quantification of endogenous compounds typically relies on the stable isotope standards. But only a few ribonucleotide standards are commercially available and these stable isotope ribonucleotide standards are normally expensive. In this respect, a pair of light and heavy stable isotope labeling reagents should be desired for the sensitive and accurate detection of endogenous ribonucleotides.

In this work, we synthesized a pair of diazo reagent of 2-(diazomethyl)-*N*-methyl-*N*-phenyl-benzamide (2-DMBA) and its counterpart of stable isotope reagent of d_5-2-(diazomethyl)-*N*-methyl-*N*-phenyl-benzamide (d_5-2-DMBA). 2-DMBA and d_5-2-DMBA can efficiently and specifically label ribonucleotides through the reaction between the diazo group on 2-DMBA or d_5-2-DMBA and phosphate group on ribonucleotides under mild conditions. With the diazo reagent labeling strategy in conjugation with mass spectrometry analysis, we

developed a highly sensitive method for determination of endogenous ribonucleotides from living organism. And we also achieved the distinct detection of endogenous ribonucleotides from only a few cells by virtue of the high detection sensitivity of the method.

EXPERIMENTAL SECTION

Chemicals and reagents

Ribonucleotide standards, including adenosine 5'-monophosphate (AMP), uridine 5'-monophosphate (UMP), cytidine 5'-monophosphate (CMP), guanosine 5'-monophosphate (GMP), adenosine 5'-diphosphate (ADP), uridine 5'-diphosphate (UDP), cytidine 5'-diphosphate (CDP), guanosine 5'-diphosphate (GDP), adenosine 5'-triphosphate (ATP), uridine 5'-triphosphate (UTP), cytidine 5'-triphosphate (CTP) and guanosine 5'-triphosphate (GTP) (Table S1 in the Supporting Information) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI), 1-hydroxybenzotriazole (HOBt), 2-carboxy benzaldehyde, *N*-methylaniline, D-chloroform ($CDCl_3$) and d_5-aniline were supplied by Sigma-Aldrich (St. Louis, MO, USA). Formaldehyde, activated manganese dioxide (MnO_2) and sodium cyanoborohydride ($NaCNBH_3$) were obtained from J&K Chemical (Beijing, China). Dimethyl sulfoxide (DMSO), hydrazine hydrate ($N_2H_4 \cdot H_2O$), dichloromethane (DCM) and ammonium bicarbonate (NH_4HCO_3) were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China).

Cell culturing and tissues

HEK293T and HeLa cell lines were obtained from the China Center for Type Culture

Collection (Wuhan, China). Dulbecco's Modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Thermo-Fisher Scientific, Waltham, MA, USA) was used for the cell culturing. Cells were maintained in a humidified atmosphere with 5% CO_2 at 37°C. Sixteen breast carcinoma tissues and matched tumor-adjacent normal tissues from 8 breast cancer patients were collected from the Hubei Cancer Hospital (Wuhan, China). An approval was granted by the Hubei Cancer Hospital Ethics Committee. All experiments were conducted in accordance with the guidelines and regulations of the Ethics Committee of the Hubei Cancer Hospital.

Sample pretreatment

HEK293T and HeLa cells were collected by centrifugation at 1500 g under 4°C for 5 min and then washed twice with ice-cold phosphate-buffered saline (PBS) to remove the medium and fetal bovine serum. The extraction of the endogenous ribonucleotides was carried out according to previous described procedure.[23] Briefly, harvested cells were quenched with prechilled 80% aqueous methanol (0°C, 2 mL) and incubated at −20°C for 30 min. Then the cell extracts were centrifuged at 14000 g at 4°C for 15 min to remove precipitated proteins. The supernatant was collected and then dried under nitrogen gas and stored at −80°C.

As for the extraction of endogenous ribonucleotides from tissues, the tissue samples (~20 mg for each one) were homogenized in prechilled 80% aqueous methanol (0°C, 1 mL). After centrifugation at 14000 g at 4°C for 15 min to remove precipitated proteins, the supernatant was collected and dried under nitrogen gas and stored at −80°C. The concentrations of protein were measured using the bicinchoninic acid (BCA) protein assay kit (Beyotime, Shanghai, China) according to the manufacturer's recommended protocol.

Synthesis of diazo reagents

The synthesis route for 2-(diazomethyl)-*N*-methyl-*N*-phenyl-benzamide (2-DMBA) was shown in Figure 1A. Briefly, 2-carboxybenzaldehyde (225 mg), EDCI (580 mg) and HOBt (500 mg) were successively added to 15 mL DCM. After stirring for 30 min, *N*-methylaniline (107 mg) was added and stirred at 25°C for 12 h to obtain 2-formyl-*N*-methyl-*N*-phenylbenzamide (2-FMBA). The 2-FMBA was purified by silica gel column chromatography (hexane/ethyl acetate: 5:1, V/V). Then $N_2H_4 \cdot H_2O$ (250 mg) was added to the purified 2-FMBA (dissolved in ethanol solution). The mixture was stirred at 70°C for 8 h followed by evaporation with vacuum to remove ethanol. At last, MnO_2 (435 mg) was added and stirred at 25°C for 4 h. The solid was filtered off and purified by silica gel column chromatography (hexane/ethyl acetate: 3:1, V/V) to obtain pure 2-DMBA. The synthesized 2-DMBA was then dissolved in DMSO and stored in -20°C.

The synthesis of d_5-2-(diazomethyl)-*N*-methyl-*N*-phenyl-benzamide (d_5-2-DMBA) was similar to that for 2-DMBA except that d_5-*N*-methylaniline was used to replace *N*-methylaniline (Figure 1A). Briefly, d_5-aniline (112 mg), formaldehyde (20 mg), and $NaCNBH_3$ (63 mg) were added to 10 mL methanol. The solution was stirred at 25°C for 12 h to obtain d_5-*N*-methylaniline. Then the following synthesis procedure was the same as that for 2-DMBA by using the synthesized d_5-*N*-methylaniline instead of *N*-methylaniline. The synthesized 2-DMBA and d_5-2-DMBA were characterized by high-resolution mass spectrometry analysis and nuclear magnetic resonance (NMR) analysis.

Chemical labeling of ribonucleotides with 2-DMBA/d_5-2-DMBA

Two hundred microliters of 50 mM borate buffer and 2 μL of certain concentrations of 2-

DMBA (d_5-2-DMBA) were added to the ribonucleotides. The reaction solution was vortexed and then incubated at different temperature and different times to optimize the reaction conditions. 2-DMBA (d_5-2-DMBA) labeled ribonucleotides were subjected to LC-ESI-MS/MS analysis.

LC-ESI-MS/MS Analysis

The LC-ESI-MS/MS analysis was performed on a system consisting of a Shimadzu 8045 mass spectrometer (Kyoto, Japan) with an electrospray ionization source (Turbo Ionspray) coupled with a Shimadzu LC-30AD UPLC system (Tokyo, Japan). The chromatographic separation of 2-DMBA or d_5-2-DMBA labeled ribonucleotides was performed on a Shimadzu Shim-pack GIST C18 column (100 mm × 2.1 mm i.d., 2.0 μm) at 35°C. A 30-min gradient with the use of 5 mM NH_4HCO_3 in H_2O (A) and methanol (B) as the mobile phases was employed for the separation of the derivatives. The flow rate was set at 0.3 mL min^{-1}. The gradient of 0−5 min 5% B, 5−15min 5 to 35% B, 15−17 min 35 to 80% B, 17−20 min 80% B, 20−23 min 80 to 5% B, and 23-30 min 5% B was used.

The native ribonucleotides and 2-DMBA/d_5-2-DMBA labeled ribonucleotides were detected by multiple reaction monitoring (MRM) in positive mode. The optimal conditions for the ionization source were as follows: ion spray voltage 4000 V, DL temperature 250°C, heat block temperature 400°C, interface temperature 300°C, nebulizing gas 2 L min^{-1}, drying gas 10 L min^{-1}, and heating gas 10 L min^{-1}. The MRM mass spectrometric parameters were optimized by direct infusion and the optimized conditions can be found in Table S2 in Supporting Information.

RESULTS AND DISCUSSION

Characterizations of synthesized 2-DMBA and d_5-2-DMBA

We first synthesized the labeling reagents of 2-DMBA and d_5-2-DMBA. The synthesis routes were shown in Figure 1A. The high-resolution mass spectrometry analysis showed two distinct ions of *m/z* 224.1065 and *m/z* of 242.1175 were observed (Figure S1A in supporting information). The ion of *m/z* 224.1065 can be assigned for $C_{15}H_{13}N_3O$ ($[M - N_2 + H]^+$, theoretical *m/z* 224.1070), while the ion of *m/z* 242.1175 can be assigned for $C_{15}H_{13}N_3O$ ($[M - N_2 + H_2O + H]^+$, theoretical *m/z* 242.1176), indicating the sucessful synthesis of 2-DMBA. Similarly, the detected ions are identical to the theoretical ions for the synthesized d_5-2-DMBA (Figure S1B in supporting information), demonstrating the sucessful synthesis of d_5-2-DMBA. Moreover, there were no detectable 2-DMBA in the synthezied d_5-2-DMBA (Figure S1B in supporting information).

In addition to MS-based charictrization, NMR analysis was also carried out to confirm the synthesized compounds of 2-DMBA and d_5-2-DMBA. The NMR spectra were shown in Figure S2-S5 in Supporting Information. ^{1}H NMR (400M Hz, $CDCl_3$): for 2-FMBA, δ 10.04 (s, 1H), 7.66 (d, J = 7.5 Hz, 1H), 7.39 –7.29 (m, 2H), 7.23 (d, J = 7.4 Hz, 1H), 7.17 – 7.02 (m, 3H), 6.98 (d, J = 7.5 Hz, 2H), 3.52 (s, 3H); for d_5-2-FMBA, δ 10.05 (s, 1H), 7.68 (d, J = 7.4 Hz, 1H), 7.39 (t, J = 7.1 Hz, 1H), 7.33 (t, J = 7.3 Hz, 1H), 7.24 (d, J = 7.5 Hz, 1H), 3.54 (s, 3H); for 2-DMBA, δ 7.20 (d, J = 7.1 Hz, 2H), 7.16 – 7.09 (m, 2H), 7.04 (d, J = 6.3 Hz, 2H), 6.97 – 6.88 (m, 1H), 6.84 (d, J = 7.8 Hz, 1H), 6.72 (s, 1H), 5.41 (s, 1H), 3.47 (s, 3H); for d_5-2-DMBA, δ 7.13 (t, J = 7.2 Hz, 1H), 6.92 (d, J = 6.4 Hz, 1H), 6.84 (d, J = 7.8 Hz, 1H), 6.72 (t, J = 7.2 Hz, 1H), 5.40 (s, 1H), 3.47 (s, 3H) (Figure S2-S5 in Supporting Information). NMR analysis demonstrated that

the desired reagents of 2-DMBA and d_5-2-DMBA were successfully synthesized.

Identification of 2-DMBA and d_5-2-DMBA labeled ribonucleotides

The chemical labeling of ribonucleotides by 2-DMBA was through the reaction of the terminal hydroxyl group of phosphate in ribonucleotides with the diazo group in 2-DMBA (Figure 1B). To examine whether the reaction was successful, we first carried out the high-resolution mass spectrometry analysis of 2-DMBA and d_5-2-DMBA labeled ribonucleotides. The extracted ion chromatograms and full scan MS spectra of 2-DMBA/d_5-2-DMBA labeled ribonucleotides are shown in Figure S6 in Supporting Information.

We also examined the MS/MS of the 2-DMBA/d_5-2-DMBA labeled ribonucleotides with collision-induced dissociation (CID) mode. Taken the CTP, CDP, and CMP as the example, it can be seen that the fragment ions generated by the 2-DMBA/d_5-2-DMBA labeled ribonucleotides (*m/z* shown in blue) were identical to their corresponding theoretical values (*m/z* shown in red, Figure 2), indicating the successful labeling of CTP, CDP, and CMP by 2-DMBA (Figure 2A) or d_5-2-DMBA (Figure 2B). It is worth noting that 2-DMBA or d_5-2-DMBA only reacts with one terminal hydroxyl group of phosphate since we didn't observe the doubly or triply labeled ribonucleotides by 2-DMBA or d_5-2-DMBA. We reasoned that the steric hindrance effect may cause the incapability for the multiple labeling of 2-DMBA to ribonucleotides. In addition, we didn't observe the reaction between 2-DMBA with the nucleobases, indicating the high labeling selectivity of 2-DMBA toward to the terminal hydroxyl group of phosphate in ribonucleotides.

Optimization of labeling reaction conditions

To obtain high labeling efficiency, the labeling conditions, including pH of reaction buffer,

reaction temperature, reaction time and the concentration of 2-DMBA were optimized. The four native ribonucleoside 5'-triphosphates (ATP, UTP, CTP and GTP) were used as the analytes for the optimization.

The pH of reaction buffer was first optimized ranging from pH 5.0 to pH 9.0. As shown in Figure 3A, the peak areas of the derivatives decreased when pH value was higher than 8.0, indicating that alkaline environment was not suitable for the labeling reaction. Thus pH 7.0 was chosen for the reaction. Then the reaction temperature was optimized from 25°C to 50°C. The results showed that the peak areas of the derivatives were basically similar (Figure 3B). We then chose 30°C for the reaction. Next, we optimized the reaction time ranging from 15 to 150 min. As shown in Figure 3C, the peak areas of the derivatives varied slightly, indicating the reaction can be finished in a relatively short time. Thus, 30-min reaction was chosen for the chemical labeling. At last, the concentration of 2-DMBA was optimized. It can be seen that the labeling efficiencies were relatively good when the concentration of 2-DMBA was 250 mg L^{-1} (Figure 3D). Taken together, the optimal conditions of the chemical labeling reaction were carried out at pH 7.0 in borate buffer under 30°C for 30 min, and the concentration of 2-DMBA was 250 mg L^{-1}.

Under the optimized conditions, almost all of the substrates of ribonucleotides were labeled to form the products (Figure S7 in Supporting Information) and the calculated labeling efficiencies for ribonucleotides were over 95%. In addition, we also examined the endogenous substrate of ribonucleotides extracted from HEK293T cells after 2-DMBA labeling. The results showed similar labeling efficiencies were achieved as those for standards (Figure S8 in Supporting Information), indicating the high labeling efficiencies toward ribonucleotides in the

sample matrix. Moreover, we evaluated the stability of 2-DMBA labeled ribonucleotides (Figure 3E). The results showed that the peak areas of these derivatives had no obvious changes at least for 24 h, which demonstrated these derivatives were relatively stable and the developed 2-DMBA labeling method was suitable for the analysis of ribonucleotides.

2-DMBA labeling improves the detection performance of ribonucleotides

We examined the detection performance for analysis of ribonucleotides by the established 2-DMBA labeling coupled with LC-ESI-MS/MS analysis. The extracted ion chromatograms of unlabeled and labeled ribonucleotides are shown in Figure 4. The retention of unlabeled native ribonucleotides was relatively weak on the reversed-phase column due to their high polarity and negatively charged phosphate group (Figure 4A), which resulted in the poor separation resolution of these native ribonucleotides. On the contrary, 2-DMBA labeled ribonucleotides exhibited increased retention on the reversed-phase column and these 2-DMBA labeled ribonucleotides also showed improved separation resolution (Figure 4B). 2-DMBA carries two phenyl groups, which could effectively increase the hydrophobicity of these derivatives and lead to the enhanced retention and eventually improved separation resolution on reversed-phase chromatographic column.

In addition to the improved LC separation, the detection sensitivities of these ribonucleotides also increased. The measured limits of detection (LODs) of 2-DMBA labeled ribonucleotides ranged from 0.07 fmol to 0.41 fmol, while the LODs of native ribonucleotides ranged from 2.1 fmol to 22.4 fmol. It can be seen that 2-DMBA labeling led to the increase of detection sensitivities of ribonucleotides for 17-174 folds (Table 1). 2-DMBA carries the tertiary amino group that is tagged to the derivatives upon labeling, which can dramatically

increase ionization efficiencies of the derivatives during MS analysis. Therefore, the 2-DMBA labeling coupled with LC-ESI-MS/MS analysis can efficiently improve the detection performance of ribonucleotides in term of LC separation and detection sensitivities. In comparison with the previously reported methods for ribonucleotides analysis, the developed 2-DMBA labeling coupled with LC-ESI-MS/MS analysis showed the best detection sensitivities (Table S3 in Supporting Information). To the best of our knowledge, the obtained LODs of these ribonucleotides by 2-DMBA labeling are the lowest ever reported.

Method validation

We next validated the detection of ribonucleotides by the developed 2-DMBA labeling coupled with LC-ESI-MS/MS analysis. The calibration curves were prepared with using different amounts of ribonucleotides (2.5, 5, 10, 20, 50, 100, 200, 500, 1000, 2000, 5000 fmol) labeled by 2-DMBA. The fixed amounts of ribonucleotides were labeled by d_5-2-DMBA, which were used as internal standards (IS). The calibration curves were constructed by plotting peak area ratios (analytes/IS) versus the molar ratios of analytes/IS with measurements by three times. The results showed good linearities were obtained with R^2 being greater than 0.9973. The LODs and LOQs of ribonucleotides were in the range of 0.07-0.41 fmol and 0.26-1.4 fmol, respectively (Table S4 in Supporting Information).

The accuracy and precision of the method were evaluated by the relative errors (REs) as well as intra- and inter-day relative standard deviations (RSDs) at three different levels (20, 200, and 2000 fmol). As shown in Table S5, the REs and RSDs were less than 15.0 % and 15.9 %, respectively, suggesting good accuracy and precision of the developed method.

Detection of ribonucleotides in cultured human cell lines

We applied this established approach to measure the endogenous contents of ribonucleotides in HEK293T and HeLa cells. The extracted ribonucleotides were labeled by 2-DMBA, and d_5-2-DMBA labeled ribonucleotide standards were added into the samples as the internal standards to calibrate the matrix effect. The extracted ion chromatograms of 12 detected ribonucleotides from HEK293T cells were shown in Figure 5. The retention times of 2-DMBA labeled ribonucleotides from HEK293T cells, 2-DMBA labeled ribonucleotide standards, and d_5-2-DMBA labeled ribonucleotide standards were all the same (Figure 5), indicating the successful detection of endogenous ribonucleotides. The measured contents of these ribonucleotides from HEK293T and HeLa cells ranged from 130 to 3000 pmol/10^6 cells (Table S6 in Supporting Information), which were comparable to the previously reported contents of ribonucleotides (Table S6 in Supporting Information). These results also demonstrated that the developed method had good detection accuracy.

Since the developed method showed high detection sensitivities for ribonucleotides, we next evaluated the possibility of this method for the analysis of endogenous ribonucleotides from only a few cells. It can be seen that all the 12 ribonucleotides (except CTP) can be distinctly detected from only 8 cells (Figure 6), suggesting the capability of the developed method on analysis of ribonucleotides from small number of cells. The high detection sensitivities for ribonucleotides make it possible to explore the endogenous ribonucleotides from rare cells, such as circulating tumor cells, in the future study.

Contents change of ribonucleotides in carcinoma tissues

Nucleotides analogues and their related phosphorylated metabolites play critical roles in tumor cell metabolism.[2] In this study, we asked whether the levels of these ribonucleotides

differ between human breast carcinoma tissues and tumor-adjacent normal tissues. We applied this method to measure the contents of 12 ribonucleotides in breast carcinoma tissues and in tumor-adjacent normal tissues. In this respect, 16 breast carcinoma tissues and matched tumor-adjacent normal tissues from 8 breast cancer patients were analyzed. The results showed that the contents of most of the ribonucleotides in breast carcinoma tissues were higher than those in tumor-adjacent normal tissues (Figure 7). We reasoned that cancer cells generally grow faster and require more of the building blocks for the synthesis of RNA,[24] which may lead to the higher contents of ribonucleotides in breast carcinoma tissues than in matched tumor-adjacent normal tissues. The detailed mechanism of the increased contents of ribonucleotides in cancer tissues needs further investigation. Nevertheless, this is the first report that ribonucleotides exhibited different levels between breast carcinoma tissues and matched tumor-adjacent normal tissues, which may also be used as the indicator of human breast cancer.

CONCLUSION

In this work, we synthesized a pair of diazo reagents of 2-DMBA and d_5-2-DMBA that can efficiently and specifically label ribonucleotides through the reaction between the diazo group on 2-DMBA/d_5-2-DMBA and phosphate group on ribonucleotides. With the synthesized diazo reagents, we established highly sensitive method for detection of endogenous ribonucleotides from living organisms by 2-DMBA/d_5-2-DMBA labeling in conjugation with mass spectrometry analysis. This developed method enables the distinct detection of endogenous ribonucleotides from only a few cells. In addition, we found the significant increase of the majority of the detected ribonucleotides in human breast carcinoma tissues compared to tumor-adjacent normal tissues. This high detection sensitivities for ribonucleotides make it possible to explore the endogenous ribonucleotides from rare circulating tumor cells in the

future study.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI:xxxxx. High-resolution mass spectrometry analysis; preparation of samples with a few cells; Table S1- S6; Figure S1- S8.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Nordlund, P.; Reichard, P. *Annu Rev Biochem* **2006**, *75*. 681-706.

(2) Jordheim, L. P.; Durantel, D.; Zoulim, F.; Dumontet, C. *Nat Rev Drug Discov* **2013**, *12*. 447-464.

(3) Friedecky, D.; Tomkova, J.; Maier, V.; Janost'akova, A.; Prochazka, M.; Adam, T. *Electrophoresis* **2007**, *28*. 373-380.

(4) Huang, D.; Zhang, Y.; Chen, X. *J Chromatogr B* **2003**, *784*. 101-109.

(5) Rajagopal, V.; Lorsch, J. R. *Methods Enzymol* **2013**, *533*. 325-334.

(6) Cohen, S.; Jordheim, L. P.; Megherbi, M.; Dumontet, C.; Guitton, J. *J Chromatogr B* **2010**, *878*. 1912-1928.

(7) Soga, T.; Ishikawa, T.; Igarashi, S.; Sugawara, K.; Kakazu, Y.; Tomita, M. *J Chromatogr A* **2007**, *1159*. 125-133.

(8) Neubauer, S.; Rugova, A.; Chu, D. B.; Drexler, H.; Ganner, A.; Sauer, M.; Mattanovich, D.; Hann, S.; Koellensperger, G. *Anal Bioanal Chem* **2012**, *404*. 799-808.

(9) Dudley, E.; Bond, L. *Mass Spectrom Rev* **2014**, *33*. 302-331.

(10) Hofmann, U.; Heinkele, G.; Angelberger, S.; Schaeffeler, E.; Lichtenberger, C.; Jaeger, S.; Reinisch, W.; Schwab, M. *Anal Chem* **2012**, *84*. 1294-1301.

(11) Zur Nedden, S.; Eason, R.; Doney, A. S.; Frenguelli, B. G. *Anal Biochem* **2009**, *388*. 108-114.

(12) Magdenoska, O.; Martinussen, J.; Thykaer, J.; Nielsen, K. F. *Anal Biochem* **2013**, *440*. 166-177.

(13) D. Garcı´a-Go´mez; E. Rodrı´guez-Gonzalo; Carabias-Martı´nez, R. *TrAC-Trend Anal Chem* **2013**, *47*. 111-128.

(14) Qi, B. L.; Liu, P.; Wang, Q. Y.; Cai, W. J.; Yuan, B. F.; Feng, Y. Q. *TrAC-Trend Anal Chem* **2014**, *59*. 121-132.

(15) Xu, J.; Zhang, Q. F.; Zheng, J.; Yuan, B. F.; Feng, Y. Q. *TrAC-Trend Anal Chem* **2019**, *112*. 161-174.

(16) Huang, T.; Armbruster, M. R.; Coulton, J. B.; Edwards, J. L. *Anal Chem* **2019**, *91*. 109-125.

(17) Cheng, Q. Y.; Xiong, J.; Wang, F.; Yuan, B. F.; Feng, Y. Q. *Chin Chem Lett* **2018**, *29*.

115-118.

(18) Lan, M. D.; Yuan, B. F.; Feng, Y. Q. *Chin Chem Lett* **2019**, *30*. 1-6.

(19) Lai, Y.; Lu, M.; Gao, X.; Wu, H.; Cai, Z. *Environ Sci Technol* **2011**, *45*. 10720-10727.

(20) Zeng, H.; Qi, C. B.; Liu, T.; Xiao, H. M.; Cheng, Q. Y.; Jiang, H. P.; Yuan, B. F.; Feng, Y. Q. *Anal Chem* **2017**, *89*. 4153-4160.

(21) Li, Z.; Zhang, H. X.; Li, Y.; Lam, C. W. K.; Wang, C. Y.; Zhang, W. J.; Wong, V. K. W.; Pang, S. S.; Yao, M. C.; Zhang, W. *Anal Chem* **2019**, *91*. 1019-1026.

(22) Gil, A.; Siegel, D.; Bonsing-Vedelaar, S.; Permentier, H.; Reijngoud, D. J.; Dekker, F.; Bischoff, R. *Metabolomics* **2017**, *13*. 1.

(23) Lu, W.; Kimball, E.; Rabinowitz, J. D. *J Am Soc Mass Spectrom* **2006**, *17*. 37-50.

(24) Hanahan, D.; Weinberg, R. A. *Cell* **2011**, *144*. 646-674.

Figure legends.

Figure 1. Synthesis of labeling reagents and the chemical labeling reaction. (A) The synthesized routes of labeling reagents, 2-DMBA and d_5-2-DMBA. (B) The chemical labeling reaction between 2-DMBA or d_5-2-DMBA with ribonucleotides.

Figure 2. The MS spectra of fragment ions generated by 2-DMBA or d_5-2-DMBA labeled ribonucleotides. (A) 2-DMBA labeled CTP, CDP, and CMP. (B) d_5-2-DMBA labeled CTP, CDP and CMP. Shown in red are the theoretical *m/z* of the precursor ions and the fragment ions; shown in blue are the measured *m/z* of the precursor ions and the fragment ions.

Figure 3. Optimization of the chemical labeling conditions and evaluation of the stability of the 2-DMBA labeled ribonucleotides. (A) Optimization of the pH of reaction buffer. (B) Optimization of reaction temperature. (C) Optimization of reaction time. (D) Optimization of the concentrations of 2-DMBA. (E) Evaluation of the stability of the 2-DMBA labeled ribonucleotides. Three independent measurements were performed and the error bars represent the standard deviations of the mean.

Figure 4. The extracted ion chromatograms of the ribonucleotides without and with 2-DMBA labeling. (A) The extracted ion chromatograms of unlabeled ribonucleotides. (B) The extracted ion chromatograms of 2-DMBA labeled ribonucleotides. 1, CTP; 2, UTP; 3, GTP; 4, ATP; 5, CDP; 6, GDP; 7, UDP; 8, ADP; 9, GMP; 10, CMP; 11, UMP; 12, AMP.

Figure 5. Determination of endogenous ribonucleotides by 2-DMBA labeling coupled with LC-ESI-MS/MS analysis. Blue lines represent the extracted ion chromatograms of 2-DMBA labeled ribonucleotide standards; red lines represent the extracted ion chromatograms of d_5-2-DMBA labeled ribonucleotide standards; black lines represent the extracted ion chromatograms

of 2-DMBA labeled ribonucleotides detected in HEK293T cells. STD, standard.

Figure 6. Detection of endogenous ribonucleotides from a few cells. The extracted ion chromatograms of 2-DMBA labeled ribonucleotides with using different numbers of cells are present. Blue lines represent 2-DMBA labeled ribonucleotides detected from 60 cells; red lines represent 2-DMBA labeled ribonucleotides detected from 30 cells; black lines represent 2-DMBA labeled ribonucleotides detected from 8 cells. Samples with a few cells were prepared by successive dilution of large number of cells.

Figure 7. Quantification of ribonucleotides in human breast carcinoma tissues and matched tumor-adjacent normal tissues. A total of 16 tissues from 8 breast cancer patients were analyzed. Two-side paired *t*-test was performed for the statistical analysis. $p < 0.05$ was considered to have significant difference.

Table 1. The limits of detection (LODs) of 12 ribonucleotides with and without 2-DMBA labeling by LC-ESI-MS/MS analysis under optimized detection conditions.

Analytes	Unlabeled (LODs, fmol)	Labeled (LODs, fmol)	Detection limit improved folds
ATP	12.5	0.14	89
UTP	30.4	0.41	74
CTP	5.3	0.32	17
GTP	15.8	0.34	46
ADP	7.8	0.10	78
UDP	22.4	0.39	59
CDP	3.7	0.18	20
GDP	8.0	0.20	40
AMP	5.4	0.08	68
UMP	19.2	0.11	174
CMP	2.1	0.07	30
GMP	7.3	0.11	66

Figure 1.

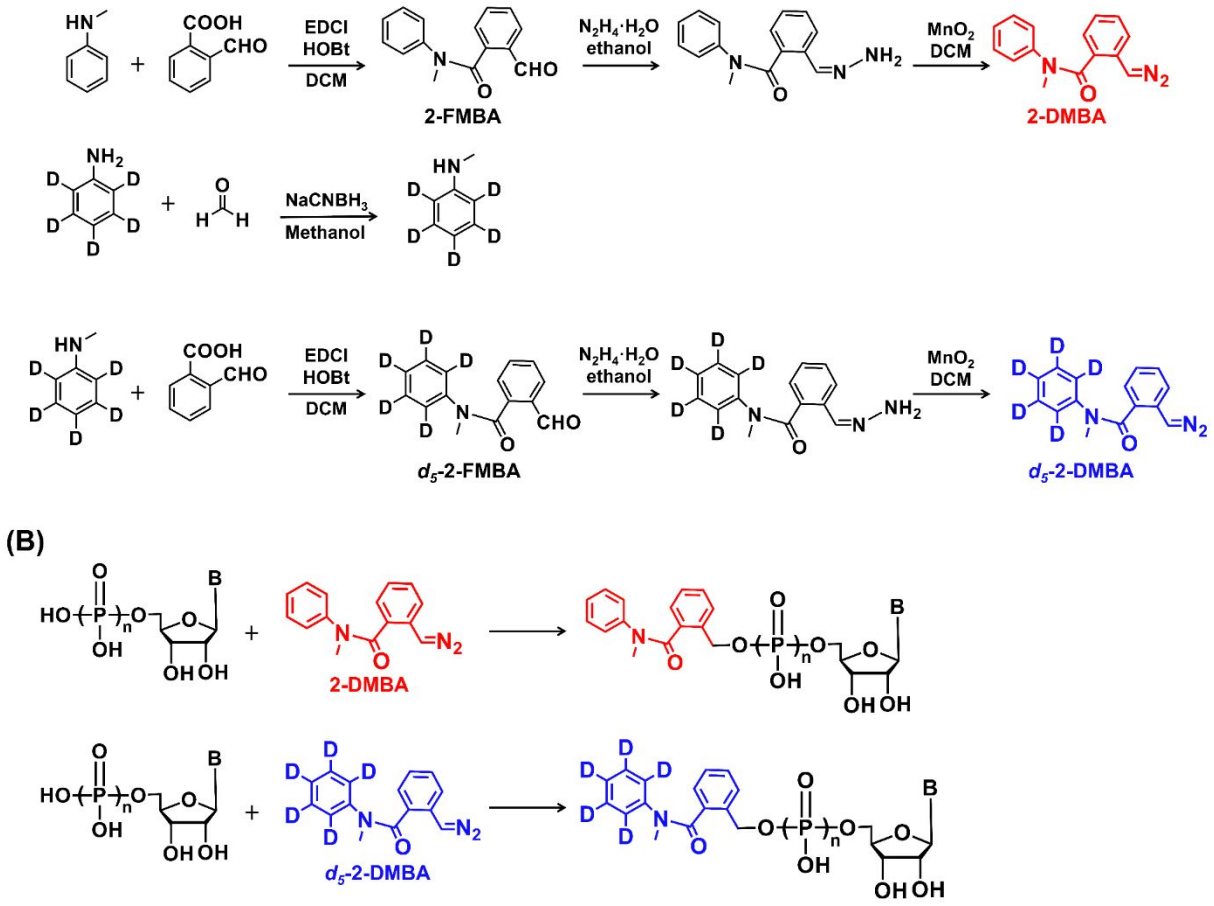

(A)
EDCI
HOBt
DCM
2-FMBA
$N_2H_4 \cdot H_2O$
ethanol
MnO_2
DCM
2-DMBA
NaCNBH$_3$
Methanol
d_5-2-FMBA
d_5-2-DMBA
(B)
2-DMBA
d_5-2-DMBA

Figure 2

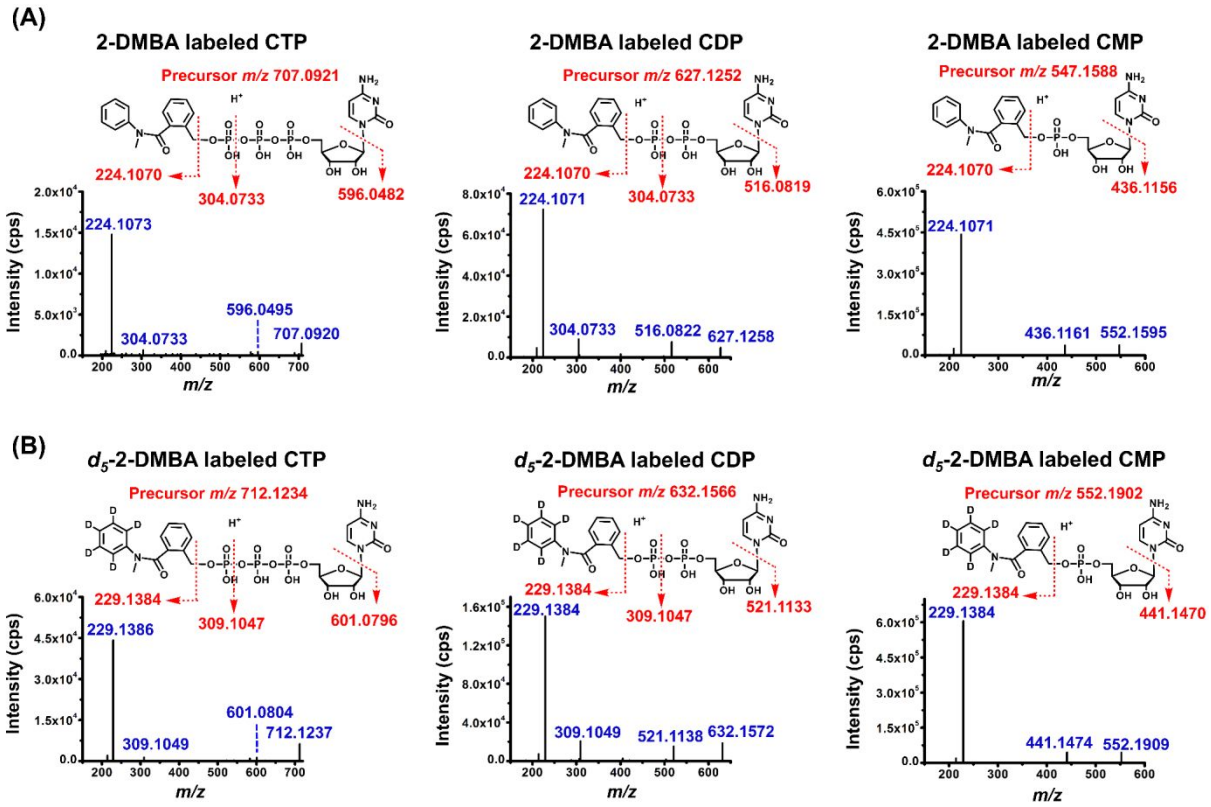

(A)
2-DMBA labeled CTP
Precursor m/z 707.0921
224.1070
304.0733
596.0482
224.1073
596.0495
304.0733
707.0920
2-DMBA labeled CDP
Precursor m/z 627.1252
224.1070
304.0733
516.0819
224.1071
304.0733
516.0822
627.1258
2-DMBA labeled CMP
Precursor m/z 547.1588
224.1070
436.1156
224.1071
436.1161
552.1595
(B)
d5-2-DMBA labeled CTP
Precursor m/z 712.1234
229.1384
309.1047
601.0796
229.1386
309.1049
601.0804
712.1237
d5-2-DMBA labeled CDP
Precursor m/z 632.1566
229.1384
309.1047
521.1133
229.1384
309.1049
521.1138
632.1572
d5-2-DMBA labeled CMP
Precursor m/z 552.1902
229.1384
441.1470
229.1384
441.1474
552.1909
Intensity (cps)
m/z

Figure 3.

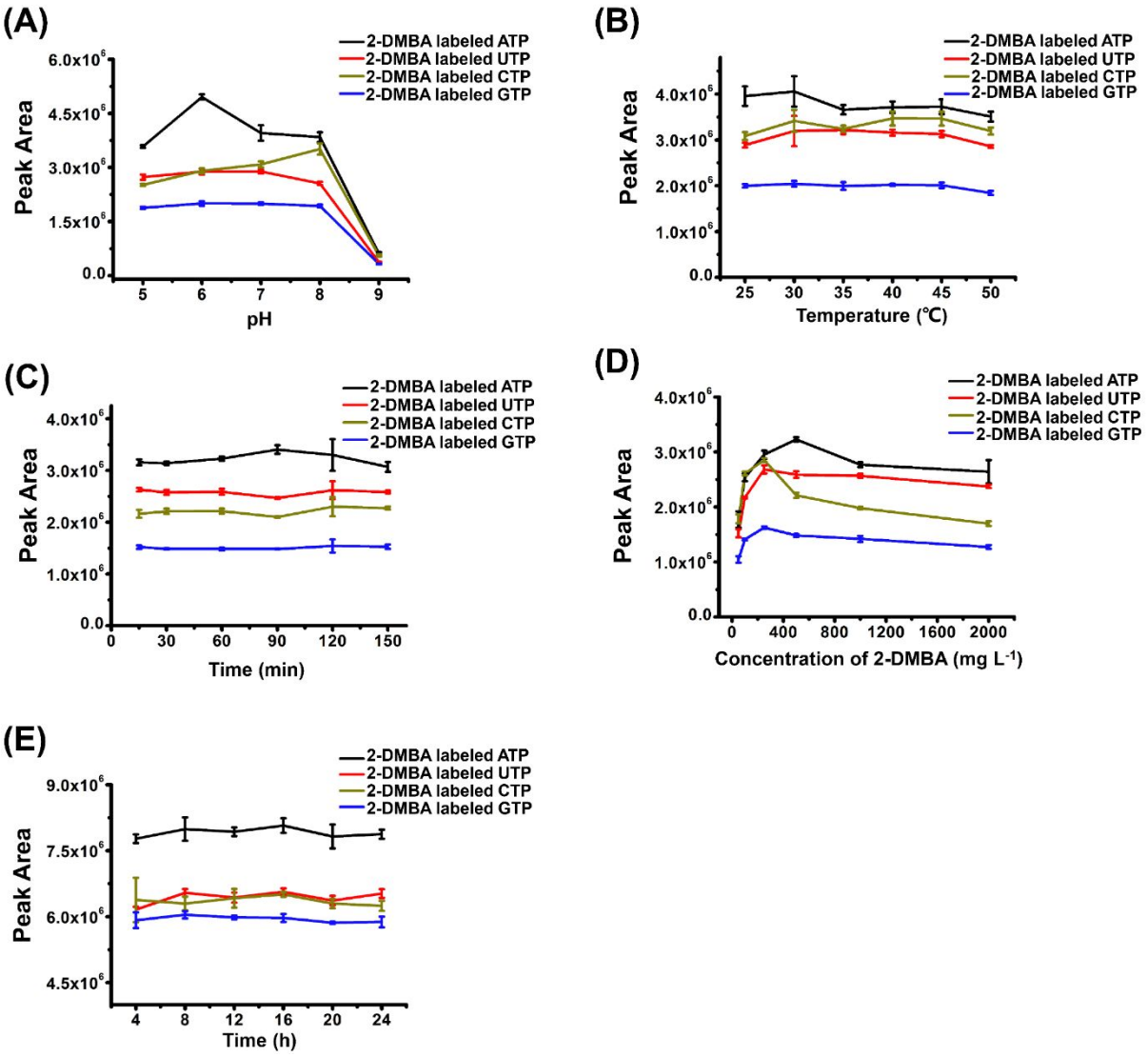

(A)
2-DMBA labeled ATP
2-DMBA labeled UTP
2-DMBA labeled CTP
2-DMBA labeled GTP
Peak Area
6.0x10^6
4.5x10^6
3.0x10^6
1.5x10^6
0.0
5
6
7
8
9
pH
(B)
2-DMBA labeled ATP
2-DMBA labeled UTP
2-DMBA labeled CTP
2-DMBA labeled GTP
Peak Area
4.0x10^6
3.0x10^6
2.0x10^6
1.0x10^6
0.0
25
30
35
40
45
50
Temperature (℃)
(C)
2-DMBA labeled ATP
2-DMBA labeled UTP
2-DMBA labeled CTP
2-DMBA labeled GTP
Peak Area
4.0x10^6
3.0x10^6
2.0x10^6
1.0x10^6
0.0
0
30
60
90
120
150
Time (min)
(D)
2-DMBA labeled ATP
2-DMBA labeled UTP
2-DMBA labeled CTP
2-DMBA labeled GTP
Peak Area
4.0x10^6
3.0x10^6
2.0x10^6
1.0x10^6
0.0
0
400
800
1200
1600
2000
Concentration of 2-DMBA (mg L-1)
(E)
2-DMBA labeled ATP
2-DMBA labeled UTP
2-DMBA labeled CTP
2-DMBA labeled GTP
Peak Area
9.0x10^6
7.5x10^6
6.0x10^6
4.5x10^6
4
8
12
16
20
24
Time (h)

Figure 4.

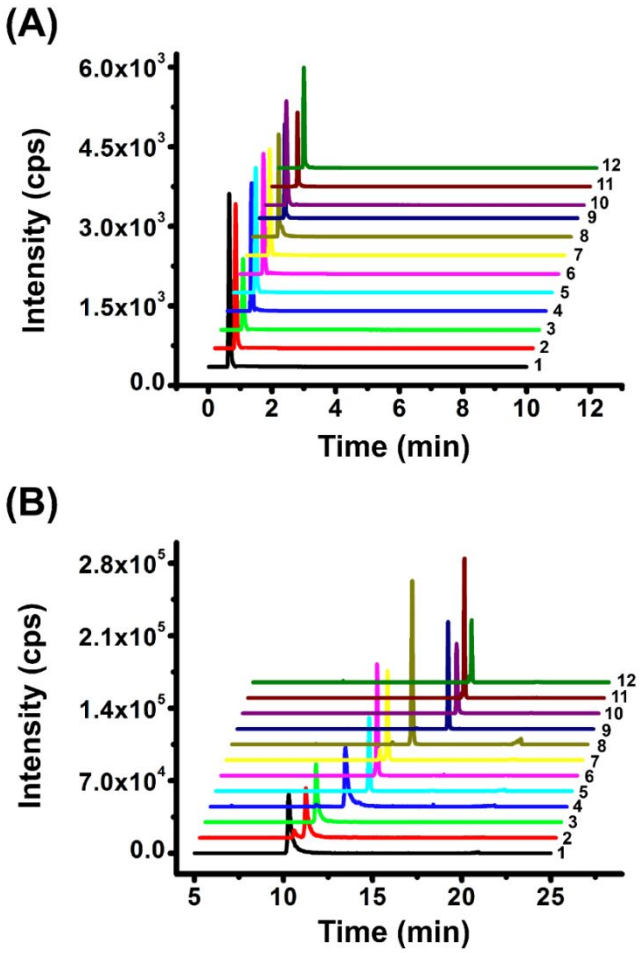

(A)
Intensity (cps)
6.0x10^3
4.5x10^3
3.0x10^3
1.5x10^3
0.0
0
2
4
6
8
10
12
Time (min)
1
2
3
4
5
6
7
8
9
10
11
12
(B)
Intensity (cps)
2.8x10^5
2.1x10^5
1.4x10^5
7.0x10^4
0.0
5
10
15
20
25
Time (min)
1
2
3
4
5
6
7
8
9
10
11
12

Figure 5.

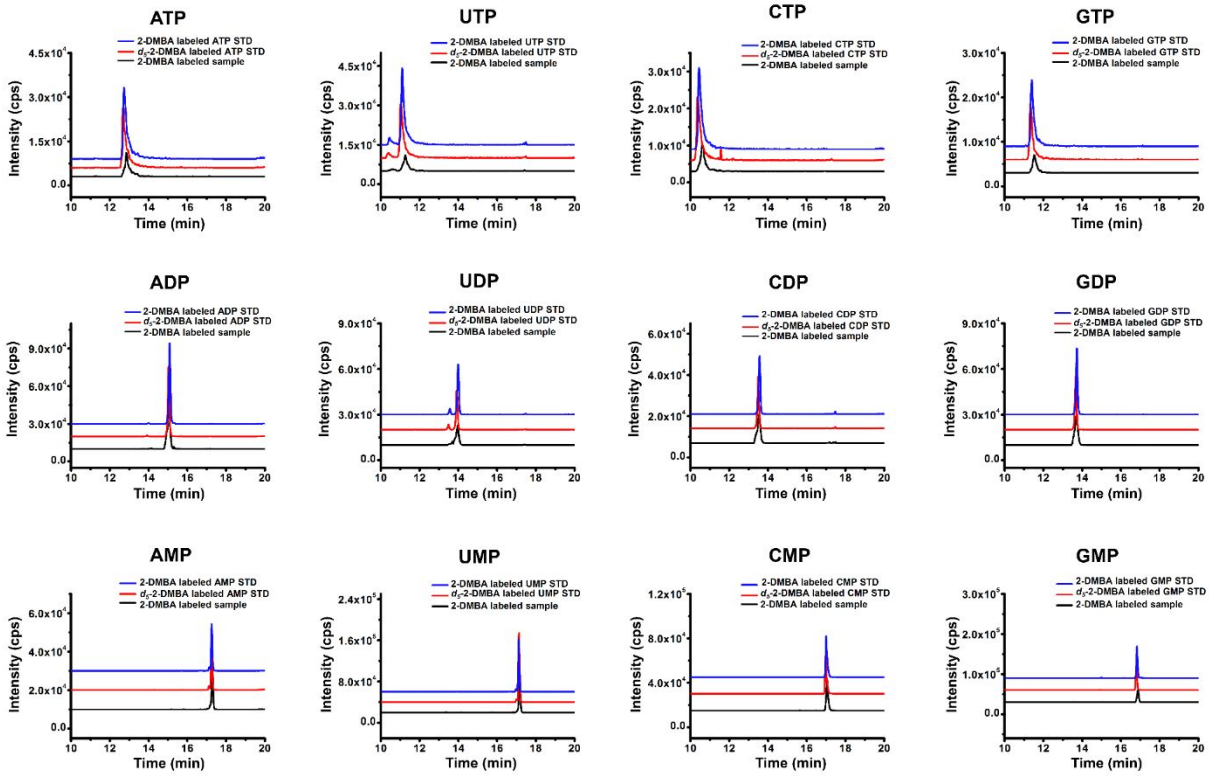

ATP
2-DMBA labeled ATP STD
d_5-2-DMBA labeled ATP STD
2-DMBA labeled sample
UTP
2-DMBA labeled UTP STD
d_5-2-DMBA labeled UTP STD
2-DMBA labeled sample
CTP
2-DMBA labeled CTP STD
d_5-2-DMBA labeled CTP STD
2-DMBA labeled sample
GTP
2-DMBA labeled GTP STD
d_5-2-DMBA labeled GTP STD
2-DMBA labeled sample
ADP
2-DMBA labeled ADP STD
d_5-2-DMBA labeled ADP STD
2-DMBA labeled sample
UDP
2-DMBA labeled UDP STD
d_5-2-DMBA labeled UDP STD
2-DMBA labeled sample
CDP
2-DMBA labeled CDP STD
d_5-2-DMBA labeled CDP STD
2-DMBA labeled sample
GDP
2-DMBA labeled GDP STD
d_5-2-DMBA labeled GDP STD
2-DMBA labeled sample
AMP
2-DMBA labeled AMP STD
d_5-2-DMBA labeled AMP STD
2-DMBA labeled sample
UMP
2-DMBA labeled UMP STD
d_5-2-DMBA labeled UMP STD
2-DMBA labeled sample
CMP
2-DMBA labeled CMP STD
d_5-2-DMBA labeled CMP STD
2-DMBA labeled sample
GMP
2-DMBA labeled GMP STD
d_5-2-DMBA labeled GMP STD
2-DMBA labeled sample
Intensity (cps)
Time (min)

Figure 6.

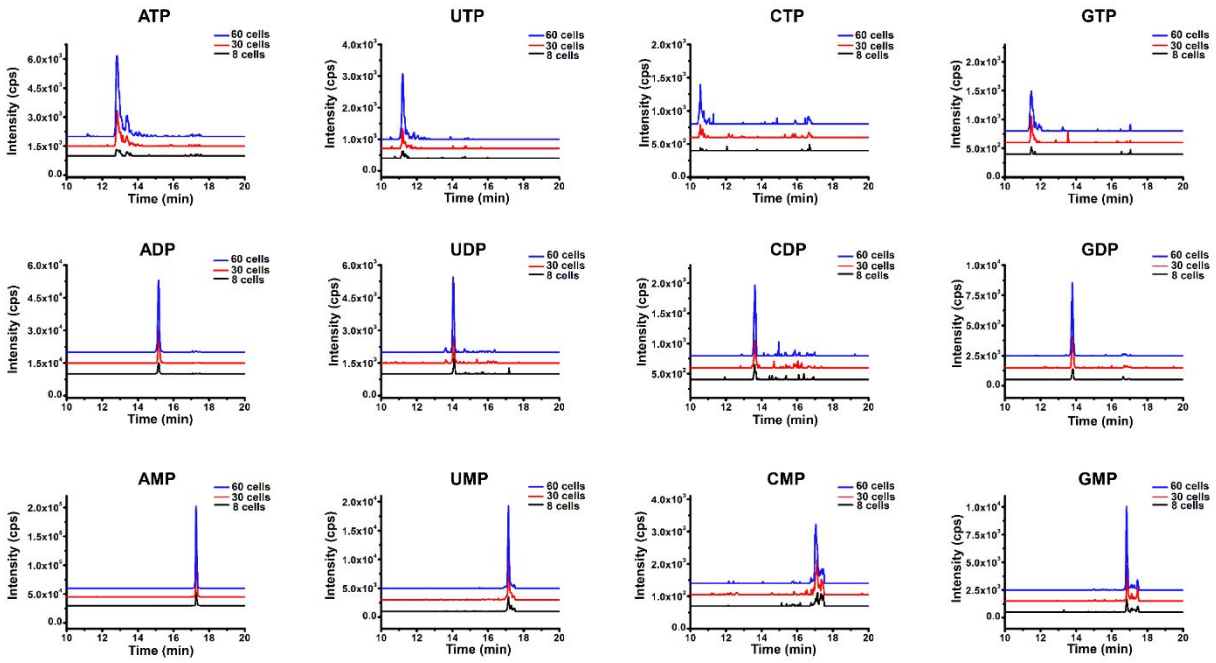
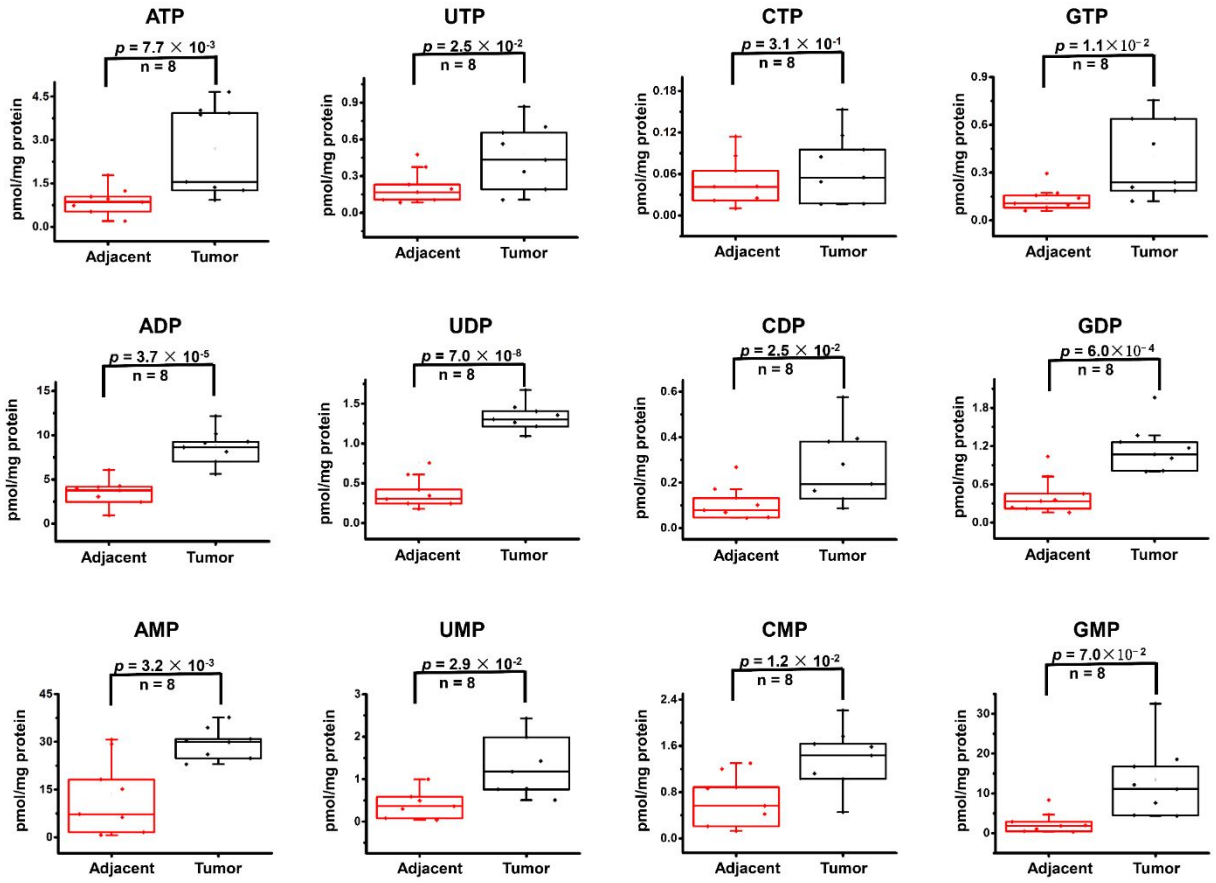
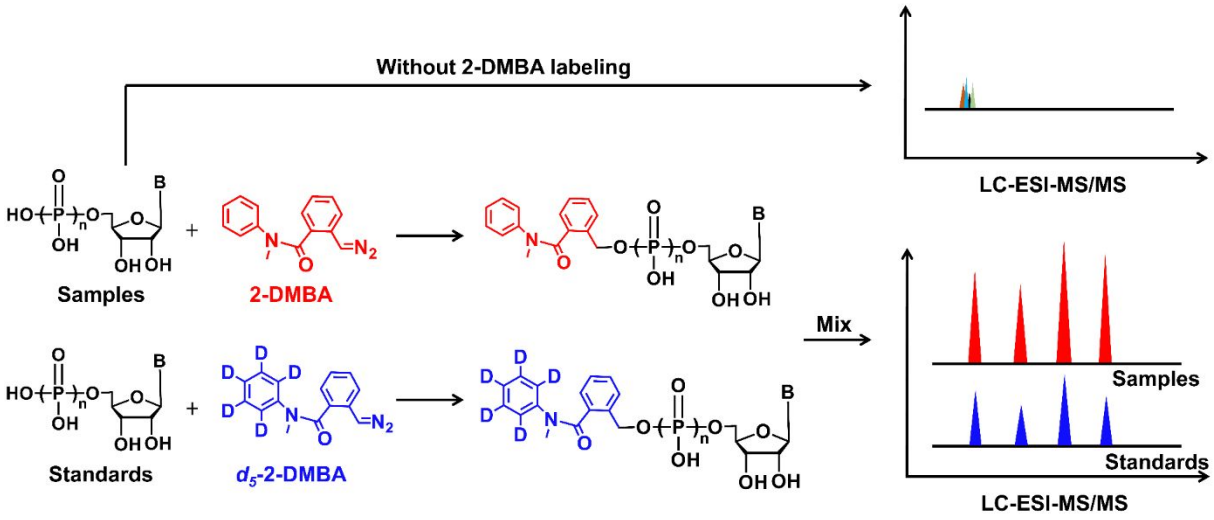

ATP
UTP
CTP
GTP
ADP
UDP
CDP
GDP
AMP
UMP
CMP
GMP
60 cells
30 cells
8 cells
Intensity (cps)
Time (min)

Figure 7.


ATP
p = 7.7 × 10-3
n = 8
UTP
p = 2.5 × 10-2
n = 8
CTP
p = 3.1 × 10-1
n = 8
GTP
p = 1.1×10-2
n = 8
ADP
p = 3.7 × 10-5
n = 8
UDP
p = 7.0 × 10-8
n = 8
CDP
p = 2.5 × 10-2
n = 8
GDP
p = 6.0×10-4
n = 8
AMP
p = 3.2 × 10-3
n = 8
UMP
p = 2.9 × 10-2
n = 8
CMP
p = 1.2 × 10-2
n = 8
GMP
p = 7.0×10-2
n = 8
pmol/mg protein
Adjacent
Tumor

TOC


Without 2-DMBA labeling
LC-ESI-MS/MS
Samples
2-DMBA
Mix
Samples
Standards
Standards
d_5-2-DMBA
LC-ESI-MS/MS