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Application News

Liquid Chromatography Mass Spectrometer LCMS-8060

Label-free quantification of RNA methylations using targeted mass spectrometry

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User Benefits

- Innovative approach for epitranscriptomics studies
- Reliable quantification of RNA Methylation
- Investigation of RNA Methylation and its impact on cellular functions such as survival, growth, and differentiation

■ Introduction

Epitranscriptomics - the study of RNA modifications - is an emerging and promising research field. RNA modifications are involved in all steps of post-transcriptional regulation of gene expression (RNA structure, splicing, export; stability, cellular localization and translation). Finally, theses modifications are essential for key cellular functions such as survival, growth and differentiation.

Here we describe an experimental workflow for the quantification of methylated nucleosides from cellular mRNA based on separation and detection on a LCMS-8060 system.

In total, more than 170 RNA modifications - including RNA methylation - have been described. These modifications are present observed on all common RNA types (mRNA, tRNA, rRNA and, ncRNA).

The most common and abundant modifications on RNA in eukaryotes is N^6-methyladenosine (m^6A). m^6A is important for mRNA regulation in physiological condition. As such, any dysregulation of m^6A level may lead to disease [1]. This modification is not limited to mRNA but is also observed in other RNA species (rRNA, tRNA, snRNA).

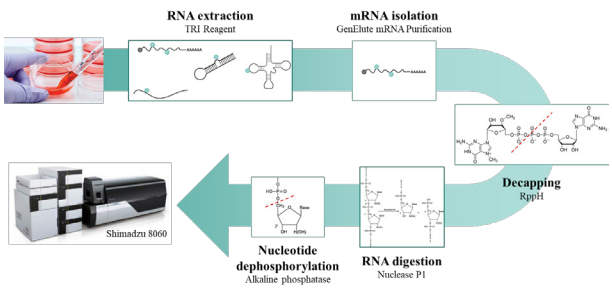

Figure 1. Workflow for label-free quantitation of RNA methylation using LCMS-8060

Common technologies to detect m^6A modifications [2] use next-generation sequencing techniques . However, these approaches cannot permit multiplex analysis of RNA methylations from a single sample. In addition, they do note enable precise quantification of individual chemical mark.

Mass spectrometry coupled to liquid chromatography has become a method of choice for accurately quantifying modified nucleosides from a biological sample. Here we present an application for the measurement of 14 distinct methylated nucleoside species using LC-MS/MS (Shimadzu LCMS-8060).

Table 1 Analytical conditions

LC conditions	
System	Nexera LC-40 X3
Analytical column	Phenomenex Synergi Fusion-RP C18, 250 x 2 mm, 4 µm, 80 Å
Mobile phase A	5 mM ammonium acetate, pH 5.3
Mobile phase B	Acetonitril
Gradient	0-3 min (0% B),3.-13 min (1-8% B), 13-23 min (8-40% B), 25-30 min (0% B)
Flow rate	0.4 mL/min
Oven temp.	35 °C
Autosampler temp.	4 °C
Injection Volume	5 µL
MS conditions	
System	LCMS-8060
Ionization	ESI (positive)
Nebulizing gas	2.5 L/min
Drying gas	3 L/min
Heating gas	12.5 L/min
Interface temp.	325 °C
DL temp.	225 °C
Heat block temp.	380 °C

■ Sample preparation

Experimental workflow for quantification of methylated nucleosides by LC-MS/MS is shown in Figure 1. 10^6 CTC44 cells (Circulating Tumor Cells) were seeded into 150 mm x 15 mm petri dishes and cultured for 4 days at 37 °C under humidified 5% CO_2 in high glucose DMEM supplemented with 2 mM glutamine and 10% FCS.

RNA extraction was achieved using TRIzol reagent (Invitrogen) according to the manufacturer instructions. mRNA was isolated using GenElute mRNA purification kit (Sigma) (2 times). First, 400 ng of RNA (in 20 µL final volume) was digested by 5 unit of RNA 5′ pyrophosphohydrolase (New England Biolabs) for 2 h at 37 °C with NEB buffer. Then decapped RNA was digested into nucleotides by 1 unit of nuclease P1 (Penicillium citrinum, Sigma) for 2 h at 42 °C on NH_4OAc buffer (10 mM, pH 5.3). Finally, nucleotides were dephosphorylated into nucleosides using 1 unit of alkaline phosphatase (Escherichia coli, Sigma) for 2 h at 37 °C on NH_4OAc buffer (100 mM) [3].

After adding 60 µL of phase A (ammonium acetate, pH 5.3), the samples are filtered (0.22 µm pore size, 4 mm diameter, Millipore). 5 µL of this solution was subjected to LCMS analysis.

■ Measurement Conditions

For the measurement, nucleosides were separated by reverse phase ultra-performance liquid chromatography using a Nexera LC-40-X3 system coupled to a LCMS-8060 triple-quadrupole mass spectrometer. Details of analysis conditions and MRM parameters are given in Tables 1 and 2. Monitoring MRM transitions enables the simultaneous measurement of numerous modified nucleosides. Modomics database [4] contains information on more than 150 distinct modified nucleoside species – including MRM information.

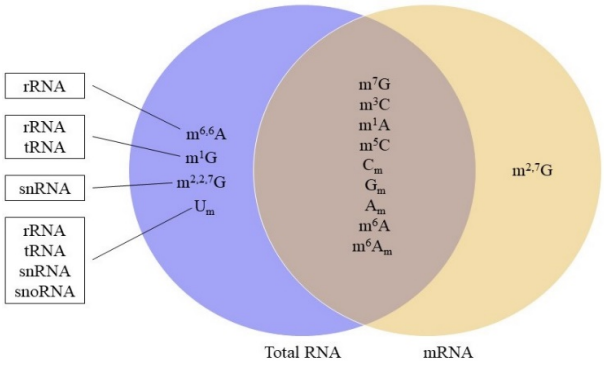

Figure 2. Distribution of the modified nucleoside species in different types of RNA. 10 nucleosides are found in mRNA. Four are merely present in other RNA types

■ Results

By quantification of methylated nucleosides from mRNA, yielded results for 10 nucleoside species from the target panel of 14 RNA methylations (Figure 3). The remaining four species have only been observed in rRNA, tRNA, and snRNA, and are to our knowledge not present in mRNA (Figure 2).

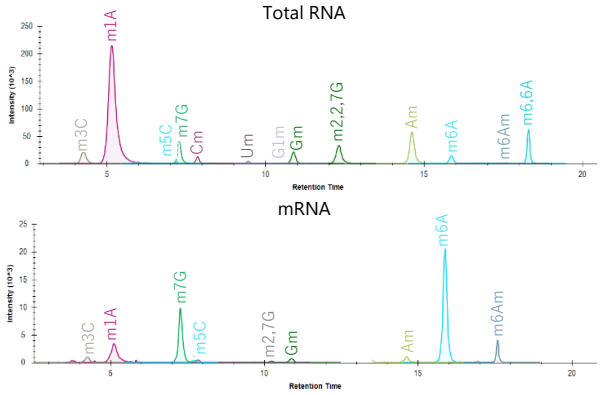

Figure 3. Extracted Ion Chromatograms of methylated nucleosides from total RNA and mRNA analyzed by LCMS

Furthermore, for most of the detected methylations, a lower signal is observed for mRNA compared to total RNA (Figure 4). For example, m^1A is found in mRNA, which accounts for only 5% of the total cell RNA, and also in tRNA and rRNA, which respectively represent 15% and 80%.

In the case of mRNA purifications, the ion signal was enhanced for methylations that are mostly predominantly found in mRNA, such as m^6A and m^6A_m (Figures 4 + 5).

Table 2 MRM parameters

Nucleoside species	Transitions	Retention time (min)	Target Dwell Time (msec)	Target Collision Energy (V)	Target Q1 Pre- Bias (V)	Target Q3 Pre- Bias (V)	Interface voltage (kV)
Adenosine (A)	268.0 > 136.0	11.8	197.0	-18.0	-24.0	-24.0	1.0
2'O-methyladenosine (A_m)	282.0 > 136.0	14.6	397.0	-17.0	-14.0	-24.0	1.5
N1-methyladenosine (m^1A)	282.1 > 150.1	5.4	197.0	-21.0	-12.0	-26.0	1.5
N6-methyladenosine (m^6A)	282.1 > 150.1	15.9	397.0	-20.0	-12.0	-16.0	1.5
N6,N6-dimethyladenosine ($m^{6,6}A$)	296.0 > 164.1	18.3	530.0	-25.0	-22.0	-20.0	1.5
N6,2'O-dimethyladenosine (m^6A_m)	296.0 > 150.0	17.6	397.0	-15.0	-18.0	-32.0	1.5
Cytidine (C)	244.1 > 112.0	3.8	197.0	-12.0	-17.0	-29.0	1.0
2'O-methycytidine (C_m)	258.1 > 112.0	7.9	142.0	-15.0	-18.0	-32.0	1.5
N3-methylcytidine (m^3C)	258.1 > 126.0	4.5	197.0	-13.0	-10.0	-14.0	1.5
N5-methylcytidine (m^5C)	258.0 > 126.0	7.3	157.0	-17.0	-14.0	-25.0	1.0
Guanosine (G)	284.1 > 152.0	8.6	142.0	-15.0	-24.0	-23.0	1.5
2'O-methylguanosine (G_m)	298.1 > 152.0	10.9	174.0	-12.0	-12.0	-17.0	1.5
N1-methylguanosine (m^1G)	298.1 > 166.0	10.6	142.0	-15.0	-11.0	-18.0	0.5
N7-methylguanosine (m^7G)	298.3 > 166.0	7.3	157.0	-10.0	-30.0	-18.0	1.5
N2,N7-dimethylguanosine ($m^{2,7}G$)	312.1 > 180.0	10.3	142.0	-12.0	-12.0	-17.0	2.5
N2,N2,N7-trimethylguanosine ($m^{2,2,7}G$)	326.15 > 194	12.4	197.0	-20.0	-15.0	-21.0	1.5
Uridine (U)	245.1 > 113.0	5.2	197.0	-11.0	-28.0	-26.0	1.0
2'O-methyluridine (U_m)	259.1 > 113.0	9.5	142.0	-9.0	-11.0	-19.0	2.5

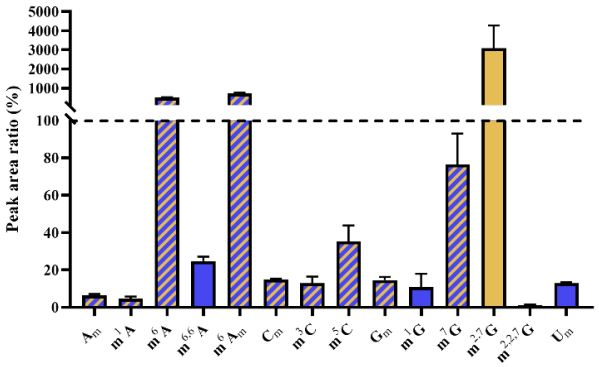

Figure 4. Level of RNA methylations in mRNA (histogram) compared to total RNA (dotted line) in CTC44 (n=3).

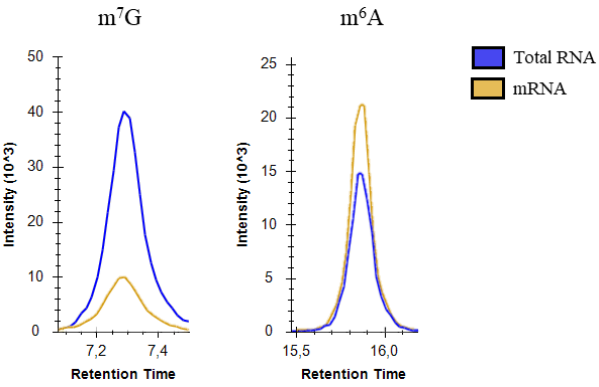

Figure 5. Overlaid extracted ion chromatograms of m^7G and m^6A in total RNA (blue line) and purified mRNA (yellow line)).

■ The Package

❑ *Main Unit*

LCMS-8060: Triple-Quad Mass spectrometer
Nexera LC-40 X3: UHPLC system

❑ *Software and Libraries*

LabSolutions LCMS
LabSolutions Insight

■ Conclusion

RNA methylations label free quantification using a triple quadrupole LC-MS/MS system (LCMS-8060 mass spectrometer coupled to Nexera LC-40 in MRM mode) represents a sensitive and reliable approach for epitranscriptomics studies.

■ References

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