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Measurement of 2-methylthio Modifications in Mitochondrial Transfer RNAs by Reverse-transcription Quantitative PCR

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[Abstract] 2-Methylthio-N^6-isopentenyladenosine (ms^2i^6A) is an evolutionally conserved posttranscriptional modification found at position 37 of four mammalian mitochondrial tRNAs, mt-$tRNA^{Ser(UCN)}$, mt-$tRNA^{Trp}$, mt-$tRNA^{Phe}$ and mt-$tRNA^{Tyr}$. The ms^2 modification in ms^2i^6A strengthens codon-anticodon interaction and contributes to accurate and efficient decoding. Deficiency of ms^2 modifications impairs mitochondrial protein synthesis, which ultimately leads to the development of myopathy in mice and patients having mitochondrial diseases. Therefore, the level of ms^2 could be utilized as an indicator that reflects the status of mitochondrial protein synthesis. Here, we describe a simple and fast quantitative PCR-based method to measure the ms^2 level in total RNA sample.

Materials and Reagents

1. Total RNA (200 ng~1 ng)
   Note: We have been using 50~200 ng RNA as the starting materials. To compare the results between experiments, it is recommended to use same amount of RNA in all experiments.
   It is recommended to use a method that can purify small RNAs [e.g. TRIzol (Invitrogen, catalog number: 15596-018) or miRNeasy Mini Kit (QIAGEN, catalog number: 217004). Check the purity of RNA by a spectrophotometer. For consistency of experiments, always use RNA with an A_{260}/A_{280} ratio > 1.8.
   Note: Currently, TRIzol is from "Thermo Fisher Scientific, Ambion™, catalog number: 15596-018".
2. PCR grade distilled water
3. Microtubes for PCR and real-time PCR (MicroAmp® Optical 8-Cap Strip) (Thermo Fisher Scientific, Applied Biosystems®, catalog number: 4323032)
4. DNase I (10x buffer is provided along with the enzyme) (Roche Diagnostics, catalog number: 04716728001)
5. Reverse transcription reagent (Transcriptor First Strand cDNA Synthesis kit) (Roche Diagnostics, catalog number: 04897030001)
   Critical Point: It is recommended to use Transcriptor First Strand cDNA Synthesis kit for measurement of ms^2 modification. Compared with other reverse transcription kits, this kit gives the highest dynamic range.

6. Real-time PCR reagent (SYBR Premix Ex Taq II) (TAKARA BIO INC., catalog number: RR820S)

   Note: SYBR green based PCR reagents from other companies work equally well.

7. Primers for detecting mitochondrial ms^2 modifications in human RNA samples
   a. Hu_tRNA^{Phe} primer f1: CTCCTCAAAGCAATACACTG
   b. Hu_tRNA^{Phe} primer r1: AGCCCGTCTAAACATTTTCA
   c. Hu_tRNA^{Phe} primer r2: GGGTGATGTGAGCCCGTCTA
   d. Hu_tRNA^{SerUCN} primer f1: GAGGCCATGGGGTTGG
   e. Hu_tRNA^{SerUCN} primer r1: CCCAAAGCTGGTTTCAAGC
   f. Hu_tRNA^{SerUCN} primer r2: AATCGAACCCCCCAAAGC
   g. Hu_tRNA^{Trp} primer f1: GGTTAAATACAGACCAAGAGC
   h. Hu_tRNA^{Trp} primer r1: CAACTTACTGAGGGCTTTGAA
   i. Hu_tRNA^{Trp} primer r2: TTAAGTATTGCAACTTACTGAGG
   j. Hu_tRNA^{Tyr} primer f1: GCTGAGTGAAGCATTGGACT
   k. Hu_tRNA^{Tyr} primer r1: AACCCCTGTCTTTAGATTTACA
   l. Hu_tRNA^{Tyr} primer r2: AGAGGCCTAACCCCTGTCTT

8. Primers for detecting mitochondrial ms^2 modification in mouse RNA samples
   a. Ms_tRNAPhe primer f1: GCTTAATAACAAAGCAAAGCA
   b. Ms_tRNAPhe primer r1: TATCCATCTAAGCATTTTCA
   c. Ms_tRNAPhe primer r2: TGGGATACAATTATCCATCT
   d. Ms_tRNA^{SerUCN} primer f1: CATATAGGATATGAGATTGGC
   e. Ms_tRNA^{SerUCN} primer r1: AACCCCCTAAAATTGGTTTCA
   f. Ms_tRNA^{SerUCN} primer r2: GAAGGAATCGAACCCCCTAA
   g. Ms_tRNA^{Trp} primer f1: GGATATACTAGTCCGCGAGC
   h. Ms_tRNA^{Trp} primer r1: GTGTTTTCTTAGGGCTTTGA
   i. Ms_tRNA^{Trp} primer r2: GTTAAACTTGTGTGTTTTCTTAG
   j. Ms_tRNA^{Tyr} primer f1: ATGGCTGAGTAAGCATTAGA
   k. Ms_tRNA^{Tyr} primer r1: ACCTCTGTGTTTAGATTTAC
   l. Ms_tRNA^{Tyr} primer r2: GAGGATTTAAACCTCTGTGT

   Notes:
   i. Standard PCR grade primers are sufficient for this protocol.
   ii. ii. The r1 primer is used for the measurement of total tRNA level, and the r2 primer is used for the measurement of ms^2-modification level in individual tRNA.

Equipment

1. Conventional PCR apparatus (Thermo Fisher Scientific, Applied Biosystems®, model: Veriti 96 Well Thermal Cycler)
2. Real-time PCR apparatus (Life Technologies, Applied Biosystems®, model: 7300 Real Time PCR System)

Note: Currently, it is "Thermo Fisher Scientific, model: 7300 Real Time PCR System".

Procedure

Part I. DNase treatment

1. Prepare total RNA from cell and tissue samples of human or animals by TRIzol reagent or miRNeasy kit according to the manufacture's instructions.
2. Mix following reagents in microtubes for DNase treatment on ice.

Component	Volume (μl/reaction)
RNA (100 ng/μl)	2
DNase I	0.5
10x DNase I buffer	2
PCR grade water	15.5
Total volume	20

Note: For measurement of ms^2 modification in multiple samples, it is recommended to prepare a master mixture of all reagents except RNA to reduce variability. When preparing a master mixture, it is usually desirable to prepare a slight excess to compensate for pipetting inaccuracies.

3. Put microtubes in PCR apparatus and use following program.

Time and temperature		
Digestion	Inactivation	Hold
37 °C for 20 min	75 °C for 10 min	4 °C

4. Use DNase I-treated sample for subsequent reverse transcription or store at -80 °C.

Part II. Reverse transcription

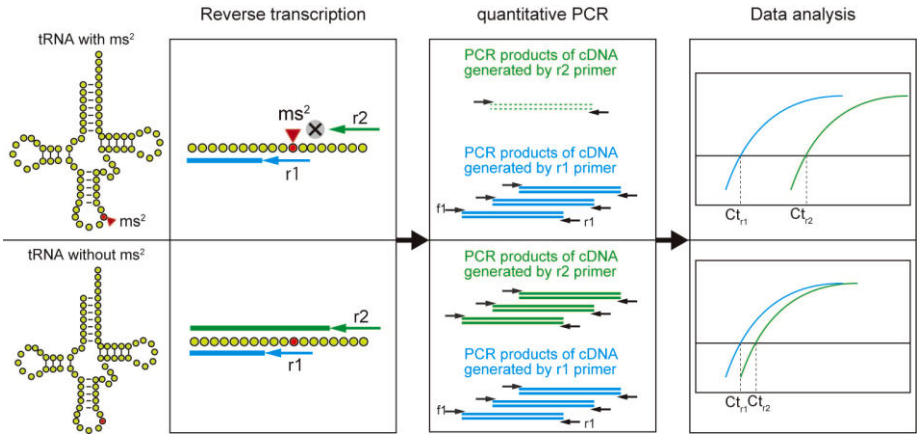

Figure 1. Workflow of the method to detect ms^2 modification. The mitochondrial tRNA is reversely transcribed by r1 primer and r2 primer, respectively. Because of the inhibitory

effect of ms^2-modification to the reverse transcription, the amount of cDNA generated by r2 primer (Green lines) highly depends on the ms^2 levels in a given RNA sample. On the other hand, the cDNA generated by r1 primer (Blue lines) is independent of ms^2 level, and could be utilized as an internal control. The amount of each cDNA is quantified by a subsequent quantitative PCR (qPCR) using f1 and r1 primers.

1. For each tRNA species, mix DNase I-digested RNA with primer r1 or primer r2 on ice.

Component	Volume (µl/reaction)	
	Sample (r1)	Sample (r2)
DNase-digested RNA	2.5	2.5
Primer r1 (10 µM)	1	—
Primer r2 (10 µM)	—	1
Water	3	3
Total volume	6.5	6.5

Note: It is recommended to prepare a master mixture of all reagents except of RNA to reduce variability. When preparing a master mixture, it is usually desirable to prepare a slight excess to compensate for pipetting inaccuracies.

2. Denature RNA at 65 °C for 10 min and immediately put samples on ice for 1 min.
3. Add RT enzyme and buffer included in the Transcriptor First Strand cDNA Synthesis kit as follows on ice.

Component	Volume (µl/reaction)	
	Sample (r1)	Sample (r2)
Denatured RNA	6.5	6.5
5x buffer	2	2
RNase inhibitor	0.25	0.25
dNTP Mix	1	1
Enzyme	0.25	0.25
Total volume	10	10

Note: It is recommended to prepare a master mixture of all reagents except of RNA to reduce variability. When preparing a master mixture, it is usually desirable to prepare a slight excess to compensate for pipetting inaccuracies.

4. Perform reverse transcription according to following program.

Time and Temperature		
Reaction	Inactivation	Hold
55 °C for 30 min	85 °C for 5 min	4 °C

5. Samples are ready for quantitative PCR. Otherwise, samples can be stored at -20 °C.

Part III. Quantitative PCR

1. Mix reagents as follows. In case other SYBR green-based PCR kits are used, follow the manufacturer' instructions as recommended.

Component	Volume (μl/reaction)	
	Sample (r1)	Sample (r2)
SYBR Premix Ex Taq	10	10
Primer f1 (10 μM)	0.4	0.4
Primer r1 (10 μM)	0.4	0.4
ROX reference dye	0.4	0.4
cDNA	2	2
Water	6.8	6.8
Total volume	20	20

Note: It is recommended to prepare a master mixture of all reagents except of cDNA to reduce variability. When preparing a master mixture, it is usually desirable to prepare a slight excess to compensate for pipetting inaccuracies.

2. Set the program as follows and run real-time PCR. Add dissociation stage if required.

Time and temperature		
First step	PCR (40 cycles)	
Heat-activation	Denaturing	Annealing/Elongation
Hold	Cycle	
95 °C for 30 sec	95 °C for 5 sec	60 °C for 31 sec

Part IV. Data analysis

Analyze data from Samples (r1) and (r2) to obtain Ct values. The results obtained Samples (r1) and (r2) represents the total tRNA level and ms^2-modification level in individual tRNA, respectively.

The normalized modification level in any given RNA sample is calculated as dCt = Ct (r2) – Ct (r1). Because the dCt value precisely reflects the modification level (Xie *et al.*, 2013), the dCt value could be directly used for comparison of modification levels between multiple samples (see below for the representative data).

Representative data

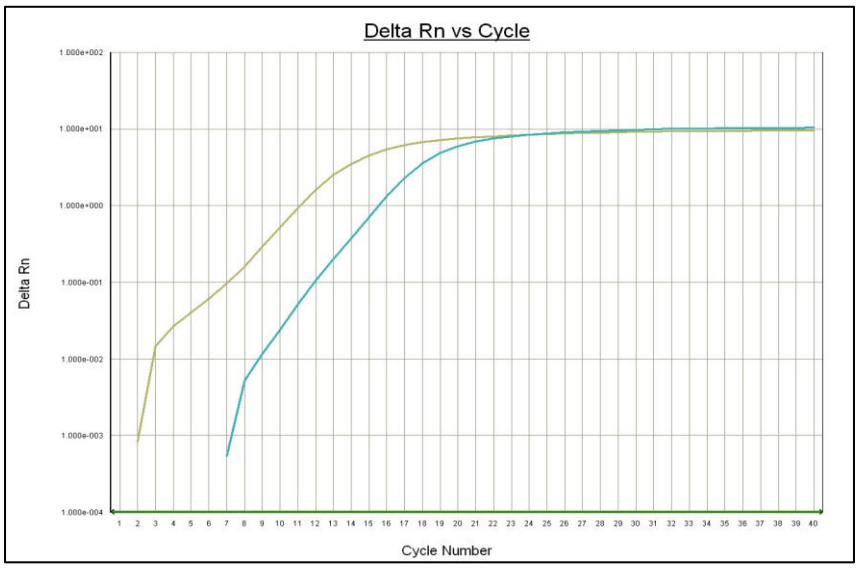

Figure 2. Measurement of ms^2 level of mt-$tRNA^{Tyr}$ in wild-type mouse heart tissue. Total RNA was purified from mouse heart tissue using TRIzol followed by 2-propanol precipitation. RNA sample was adjusted to 100 ng/µl and subjected to analysis as described above. The representative amplification curves using r1 primer (green) and r2 primer (blue) are shown. Ct (r2) = 14.8, Ct (r1) = 10.7. Modification level (dCt) in wild-type mouse heart = 14.8-10.7 = 4.1.

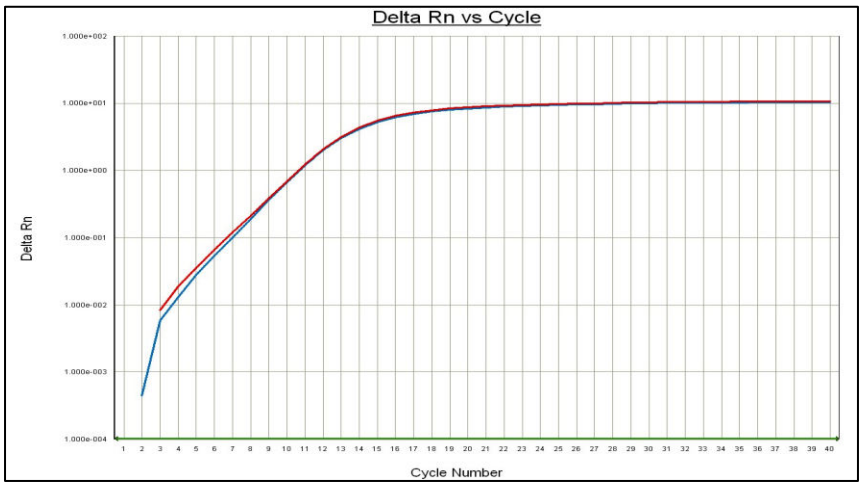

Figure 3. Measurement of ms^2 level of mt-$tRNA^{Tyr}$ in heart tissue of Cdk5rap1 KO mouse. Total RNA was purified from heart tissue of Cdk5rap1 KO mouse that does not contain ms^2 modification. RNA sample was adjusted to 100 ng/µl and subjected to analysis as described above. The representative amplification curves using r1 primer (red) and r2 primer (blue) are shown. Ct (r2) = 10.4, Ct (r1) = 10.4. Modification level (dCt) in Cdk5rap1 KO mouse heart = 10.4-10.4 = 0.

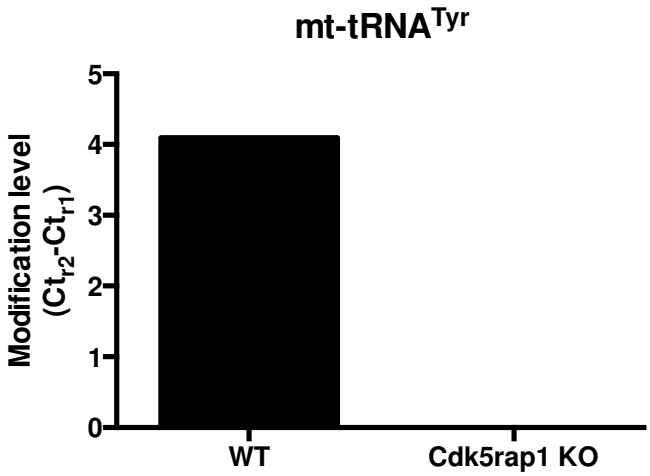

Figure 4. Comparison of the modification levels of mt-tRNA^{Tyr} in heart tissues of wild-type (WT) and Cdk5rap1 KO mouse

Acknowledgments

The concept of this protocol was adapted from our previous study, in which the quantitative PCR was used to measure ms^2 modification in cytosolic $tRNA^{Lys(UUU)}$ (Xie *et al.*, 2013). We have successfully used this protocol to measure ms^2 modification mitochondrial tRNA in our recent study (Wei *et al.*, 2015). This work was supported by a Grant-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

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