Chapter 17

Probing *N*^{6}-methyladenosine (m^{6}A) RNA Modification in Total RNA with SCARLET

Nian Liu and Tao Pan

Abstract

Posttranscriptional *N*^{6}-methyladenosine (m^{6}A) RNA modification is indispensable for cell development and viability; however, functional investigation of m^{6}A biological function has been hindered by the lack of methods for its precise identification and quantitation. Here, we describe a method that accurately identifies m^{6}A position and modification fraction in human messenger RNA (mRNA) and long noncoding RNA (lncRNA) at single-nucleotide resolution, termed as "site-specific cleavage and radioactive-labeling followed by ligation-assisted extraction and thin-layer chromatography (SCARLET)" (Fig. 1). This method combines two previously established techniques, site-specific cleavage and splint ligation, to probe the m^{6}A RNA modification status at any mRNA/lncRNA site in the total RNA pool.

Key words *N*^{6}-methyladenosine, SCARLET, RNA modification, Modification fraction, Single-nucleotide resolution, mRNA/lncRNA

1 Introduction

Discovered in the 1970s, m^{6}A is the most prevalent internal mRNA/lncRNA modification in eukaryotes, present on average in over three sites per mRNA molecule in mammals [1–6]. The m^{6}A/MeRIP-seq revealed the m^{6}A topology along mammalian transcript at ~100 nucleotides resolution [7–9]. In this chapter, we describe a protocol to determine the exact position as well as the modification fraction of mRNA/lncRNA modifications at single-nucleotide resolution without the need to isolate the target RNA. This method has been applied to reveal the m^{6}A status in human mRNA/lncRNAs, which provided information on the location and structural contexts of m^{6}A modification [10].

Erik Dassi (ed.), *Post-Transcriptional Gene Regulation*, Methods in Molecular Biology, vol. 1358,
DOI 10.1007/978-1-4939-3067-8_17,

2 Materials

Prepare all solutions using RNase-free water (prepared by autoclaving deionized water).

1. PerfectPure RNA cultured cell kit (5′).
2. GenElute mRNA miniprep kit (Sigma-Aldrich).
3. T4 PNK (USB).
4. Thermosensitive alkaline phosphatase, TAP (Thermo Scientific).
5. Crush and soak buffer, 50 mM Potassium Acetate, 200 mM KCl, pH 7.
6. RNase T1 (Thermo-Scientific).
7. RNase A (Sigma-Aldrich).
8. T4 DNA ligase (Thermo-Scientific).
9. Nuclease P1 (Sigma-Aldrich).
10. TLC cellulose plastic sheet, 20 × 20 cm (Merck).
11. TLC running buffer, isopropanol:HCl:water, 70:15:15, v/v/v.

3 Methods

The method is termed as "*s*ite-specific *c*leavage *a*nd *radioactive-labeling* followed by *l*igation-assisted *e*xtraction and *t*hin-layer chromatography (SCARLET)." SCARLET is composed of four main steps: site-specific cleavage at the target nucleotide site; radioactive labeling of the target nucleotide; splint-assisted ligation followed by RNase T1/A digestion; and thin-layer chromatography (TLC) (Fig. 1). We describe the SCARLET method in detail below in each of these four steps. Perform at room temperature (RT) unless specifically indicated.

When interested in the modification status (the presence of modification and the modification fraction) of the target nucleotide X along the target mRNA Y, we need to first design the chimeric oligos, splint oligos and ssDNA oligos according to the sequence of the target mRNA Y, as previously reported [10–14]. All chimeric oligos and DNA oligos can be ordered from IDT, and gel purified before use.

Before performing SCARLET, isolate total RNA from HeLa cells or other cell lines using PerfectPure RNA cultured cell kit (5′) according to the manual. Then, isolate polyadenylated RNA (polyA$^+$ RNA) from the total RNA sample via the GenElute mRNA miniprep kit (Sigma-Aldrich) according to the manual. Store the RNA at −80 °C until ready for SCARLET.

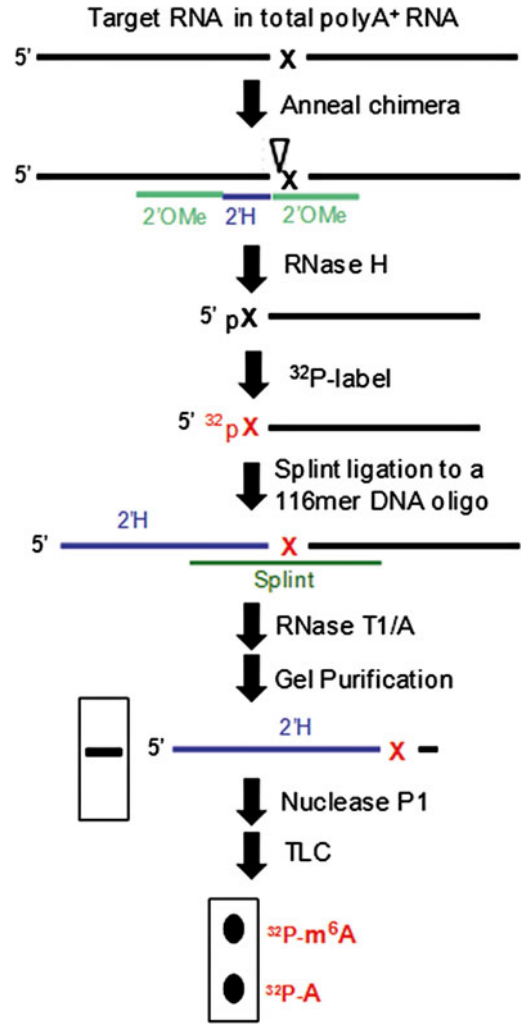

Fig. 1 Schematic diagram of SCARLET. SCARLET consists of four steps: site-specific cleavage at the target nucleotide site; radioactive labeling of the target nucleotide; splint-assisted ligation followed by RNase T1/A digestion; and thin-layer chromatography (TLC)

3.1 Site-Specific Cleavage at the Target Nucleotide Site

Day 1

1. Mix 1 μg polyA+ RNA with (3 pmol) corresponding chimeric oligo in a total volume of 3 μl 30 mM Tris–HCl, pH 7.5. Anneal the oligo to RNA by heating at 95 °C for 1 min, followed by incubation at room temperature (RT) for 3 min before putting on ice for the next step.
2. Add 1 μl 5× RNase H reaction mixture [2× T4 polynucleotide kinase buffer (T4 PNK, USB), 1 U/μl RNase H (Epicentre)] and 1 μl thermosensitive alkaline phosphatase (1 U/μl, TAP, Thermo Scientific) to the annealed RNA sample. Incubate at

44 °C for 1 h for site-specific cleavage and dephosphorylation at the 5′ end of nucleic acids.

3. Terminate the reaction by heating the reaction mixture at 75 °C for 5 min followed by immediate incubation on ice to inactivate RNase H and TAP.

3.2 Radioactive Labeling of the Target Nucleotide

1. Add 1 μl 6× T4 PNK reaction mixture [1× T4 PNK buffer, 6 U/μl T4 PNK (USB), 28 μCi/μl [γ-^{32}P]-ATP] to the mixture from above.
2. Incubate at 37 °C for 1 h.
3. Terminate the reaction by heating the mixture at 75 °C for 5 min followed by immediate incubation on ice to inactive the T4 PNK.

3.3 Splint-Assisted Ligation Followed by RNase T1/A Digestion

1. Add to the reaction mixture from above 1.5 μl the splint/ssDNA-116 oligo mixture (4 pmol splint oligos and 5 pmol ssDNA-116 oligos), and mix well.
2. Anneal the RNA samples, splint oligos, and ssDNA-116 oligos by heating the mixture at 75 °C for 3 min, followed by incubation at room temperature (RT) for 3 min before putting on ice for the next step.
3. Add 2.5 μl 4× ligation mixture [1.4× T4 PNK buffer, 0.27 mM ATP, 57 % DMSO, 1.9 U/μl T4 DNA ligase].
4. Incubate at 37 °C for 3.5 h for the splint ligation.
5. Terminate the reaction by mixing the reaction samples with equal volume of 2× RNA loading buffer (9 M urea, 100 mM EDTA, XC, and BPB dyes).
6. Add 1 μl RNase T1/A mixture (160 U/μl RNase T1, 0.16 mg/ml RNase A in distilled water), and mix well.
7. Incubate at 37 °C overnight (~16 h) to ensure complete RNase digestion.

Day 2

8. Spin down the reaction mixture. Load all samples to a pre-run 10 % urea denaturing PAGE gels (0.8 mm double-thick gel). Run the bromophenol dye to the bottom.
9. Disassemble the gel electrophoresis equipment, wrap the gel with plastic film, and expose the gel to a blanked phosphorimager screen. To get clear phosphorimaging figures with visible target bands, the exposure time varies from 10 to 30 min, depending on the radioactive signal strength (Fig. 2).
10. Visualize and print the phosphorimager figure in the actual size. Put the printed figures under the gel for localization of the target bands.

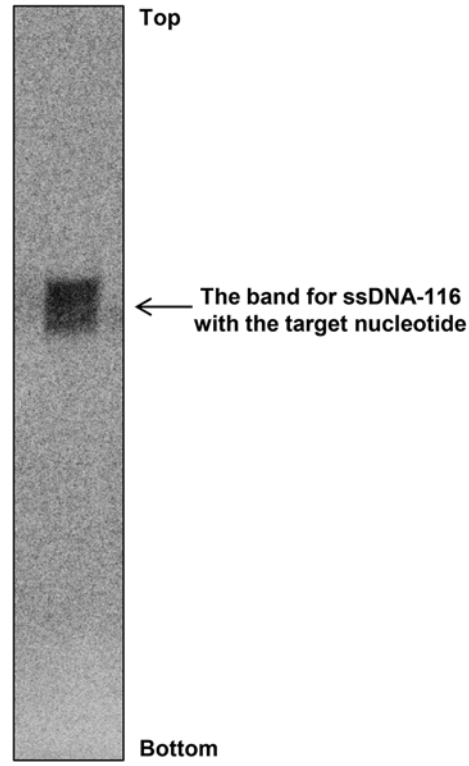

Fig. 2 Denaturing PAGE showing the band for ssDNA-116 oligos with ligated radioactive target nucleotide. This band was derived from the SCARLET experiment with the 2577-A site on the MALAT1 lncRNA (NR_002819) from HFF-1 cells

11. Cut the bands with flame-sterilized blades from the gel and transfer the cut gel slices into a clean 1.5 ml low-adhesion, plastic tube.
12. Add 0.4 ml crush and soak buffer to the tube, and invert and rotate the tube for 4 h at RT.
13. Transfer the crush and soak buffer containing the target oligos out to new low-adhesion tubes, and add 2.7× vol. of pure ethanol. Mix well, freeze at −20 °C for at least 1 h, and then precipitate the nucleic acid products in microcentrifuge at 16k×*g* for 20–30 min.
14. Vacuum or air-dry the pellet. Expect good signals for the next step when the radioactive signal is detectable by the Geiger counter.

3.4 Thin-Layer Chromatography Reveals the Modification Status

1. Resuspend and dissolve the alcohol-precipitated RNA pellet with 3 μl nuclease P1 mixture (0.33 U/μl nuclease P1 in 30 mM sodium acetate/acetic acid, pH 4.8).
2. Incubate at 37 °C for 2 h to allow complete digestion.

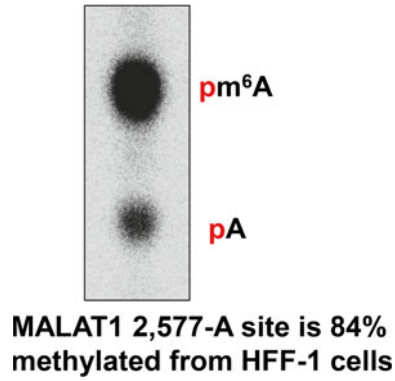

Fig. 3 TLC result showing the modification status of the 2577-A site on the MALAT1 lncRNA (NR_002819) from HFF-1 cells

3. Spot the reaction mix 0.5–1 μl at a time on a cellulose TLC plate (20 × 20 cm; Merck) as previously described. If multiple spotting is needed, wait for the TLC plate to dry completely before spotting the next aliquot and ensure spotting at the same position of the TLC plate.
4. Develop the TLC plate in a tank with running buffer [isopropanol:HCl:water (70:15:15, v/v/v), 100 ml]. This process takes ~14 h.

Day 3

5. After that, dry the TLC plate at room temperature for 1 h, wrap the plate in plastic film and expose it to a blanked phosphorimager screen. The exposure time varies from 1 to 20 h, depending on the strength of radioactive signals on the TLC plate.
6. Visualize and quantify the TLC result through the phosphorimager to get the modification status of the target nucleotide (Fig. 3). Please *see* **Notes 1–7**.

4 Notes

1. RNA modifications in abundant RNA, such as ribosomal RNA, small nuclear RNA, etc., normally generate very strong SCARLET signal.
2. RNA modifications in abundant mRNA/lncRNA tend to have stronger SCARLET signal and lower background noise signal on the TLC plate.
3. It is strongly advisable to use specific, synthetic oligos as reaction and TLC controls. Synthetic oligos normally ensure significant signal of m^{6}A on the electrophoresis gel and TLC

result, providing valuable information whether every step is done correctly, all reagents and enzymes are active, and the correct position of the bands or dots containing the target nucleotides.

4. The m^{6}A RNA modification fraction on mRNA/lncRNA normally ranges between 5 and 88 % [10]. We arbitrarily set the detection threshold at 5 %, so modification signal with a fraction less than 5 % is considered background noise or unmodified.
5. Since each SCARLET experiment works on only one candidate site, careful works on selecting candidate sites are needed to ensure successful detection of m^{6}A RNA modification. Potential candidate m^{6}A sites are evaluated through previous m^{6}A/MeRIP results, RNA abundance through previous RNA-seq data or else, RRACH consensus motif [15, 16], structural motif embedded, species-conserved level, and so on. More detailed selection process was described in ref. 10.
6. We normally perform ~10 SCARLET experiments in parallel to examine 10 potential m^{6}A sites at one time.
7. The modification fraction of m^{6}A located in structured RNAs can be underestimated by SCARLET, due to the inefficient hybridization with chimeric oligos [17].

Acknowledgments

We thank Dr. Q. Dai for assistance with chemical synthesis, and Dr. M. Parisien for bioinformatic analysis to choose potential target nucleotide sites from mRNA/lncRNA. This work was supported by a NIH grant (GM088599 to T.P. and C.H.).

References

1. Bokar JA (2005) The biosynthesis and functional roles of methylated nucleosides in eukaryotic mRNA. In: Grosjean H (ed) Fine-tuning of RNA functions by modification and editing. Berlin, Springer, pp 141–178
2. Liu N, Pan T (2014) RNA epigenetics. Am J Transl Res 165:28–35
3. Pan T (2013) N^6-methyl-adenosine modification in messenger and long non-coding RNA. Trends Biochem Sci 38:204–209
4. Horowitz S, Horowitz A, Nilsen TW, Munns TW, Rottman FM (1984) Mapping of *N6-methyladenosine* residues in bovine prolactin mRNA. Proc Natl Acad Sci U S A 81: 5667–5671
5. Dai Q, Fong R, Saikia M et al (2007) Identification of recognition residues for ligation-based detection and quantitation of pseudouridine and N^6-methyladenosine. Nucleic Acids Res 35:6322–6329
6. Fu Y, Dominissini D, Rechavi G et al (2014) Gene expression regulation mediated through reversible m^{6}A RNA methylation. Nat Rev Genet 15:293–306
7. Dominissini D, Moshitch-Moshkovitz S, Schwartz S et al (2012) Topology of the human and mouse m^{6}A RNA methylomes revealed by m^{6}A-seq. Nature 485:201–206
8. Meyer KD, Saletore Y, Zumbo P et al (2012) Comprehensive analysis of mRNA methylation reveals enrichment in 3' UTRs and near stop codons. Cell 149:1635–1646
9. Chen K, Lu Z, Wang X et al (2014) High-resolution N^6-methyladenosine (m^{6}A) map using

photo-crosslinking-assisted m A sequencing. Angew Chem. doi:10.1002/anie.201410647

10. Liu N, Parisien M, Dai Q et al (2013) Probing N^6-methyladenosine RNA modification status at single nucleotide resolution in mRNA and long noncoding RNA. RNA 19:1848–1856
11. Zhao X, Yu YT (2004) Detection and quantitation of RNA base modifications. RNA 10:996–1002
12. Maroney PA, Chamnongpol S, Souret F et al (2008) Direct detection of small RNAs using splinted ligation. Nat Protoc 3:279–287
13. Wu G, Xiao M, Yang C et al (2011) U2 snRNA is inducibly pseudouridylated at novel sites by Pus7p and snR81 RNP. EMBO J 30:79–89
14. Yu YT, Shu MD, Steitz JA (1997) A new method for detecting sites of 2'-*O*-methylation in RNA molecules. RNA 3:324–331
15. Harper JE, Miceli SM, Roberts R et al (1990) Sequence specificity of the human mRNA *N6-adenosine* methylase in vitro. Nucleic Acids Res 18:5735–5741
16. Liu J, Yue Y, Han D et al (2014) A METTL3-METTL14 complex mediates mammalian nuclear RNA N^6-adenosine methylation. Nat Chem Biol 10:93–95
17. Liu N, Dai Q, Zheng G, He C, Parisien M, Pan T (2015) N^6-methyladenosine-dependent RNA structural switches regulate RNA-protein interactions. Nature 518:560–564