Chapter 20

Detecting m⁶A with In Vitro DART-Seq

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Abstract

Recent studies have uncovered that cellular mRNAs contain a diverse epitranscriptome comprising chemically modified bases which play important roles in gene expression regulation. Among these is m^6A, which is a highly prevalent modification that contributes to several aspects of RNA regulation and cellular function. Traditional methods for m^6A profiling have used m^6A antibodies to immunoprecipitate methylated RNAs. Although powerful, such methods require high amounts of input material. Recently, we developed DART-seq, an antibody-free method for m^6A profiling from low-input RNA samples. DART-seq relies on deamination of cytidines that invariably follow m^6A sites and can be performed using a simple in vitro assay with only 50 ng of total RNA. Here, we describe the in vitro DART method and present a detailed protocol for highly sensitive m^6A profiling from any RNA sample of interest.

Key words DART-seq, m^6A, Epitranscriptome, In vitro deamination, Protein purification, RNA isolation

1 Introduction

N^6-methyladenosine (m^6A) is the most abundant internal mRNA modification and is found in thousands of cellular RNAs. Recent studies have revealed that m^6A plays diverse roles in regulating RNA function and gene expression and that it contributes to a wide range of physiological processes such as stem cell maintenance and learning and memory [1–4]. Therefore, the development of methods to profile m^6A has become increasingly important in order to understand the distribution and regulation of this mark across different cell and tissue types. Recently, we developed DART-seq (*d*eamination *a*djacent to *R*NA modification *t*argets), a novel method for global m^6A detection from low-input RNA samples [5]. This method utilizes a fusion protein consisting of the m^6A-binding YTH domain fused to the cytidine deaminase enzyme APOBEC1. When the APOBEC1-YTH protein encounters methylated RNA, it binds to the m^6A residue and converts adjacent cytidine residues that invariably follow m^6A sites into uridines.

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The resulting C to U mutations can then be detected with next-generation sequencing or simple Sanger sequencing to identify m^6A sites. This in vitro DART assay is simple, fast, and highly sensitive.

Here, we demonstrate that m^6A residues in cellular RNA samples can be detected with in vitro DART assays using as little as 50 ng of total RNA. We describe a detailed protocol for purification of the APOBEC1-YTH protein as well as a companion protein, APOBEC1-YTH^{mut}, which has diminished m^6A binding ability and is employed as a negative control to increase m^6A detection stringency. We also present detailed assay conditions and provide important notes to improve assay performance.

2 Materials

2.1 Induction of DART Protein Expression in E. coli

1. pCMV-APOBEC1-YTH (Addgene #131636).
2. pCMV-APOBEC1-YTH^{mut} (Addgene #131637).
3. pET His6 MBP TEV LIC cloning vector (Addgene #29656).
4. Rosetta™ 2 (DE3) pLysS Singles™ Competent Cells.
5. 30% glycerol, autoclaved.
6. LB medium: 1% (w/v) tryptone, 1% (w/v) NaCl, 0.5% (w/v) yeast extract, 50 μg/mL kanamycin.
7. Autoinduction medium (1 L): 958 mL ZY medium, 20 mL M medium (50×), 20 mL 5052 medium (50×), 2 mL $MgSO_4$ (1 M), 100 μg/mL kanamycin.
8. ZY medium: 1% (w/v) tryptone, 0.5% (w/v) yeast extract. Autoclave and store at 4 °C.
9. M medium (50×): 1.25 M Na_2HPO_4, 1.25 M KH_2PO_4, 2.5 M NH_4Cl, 250 mM Na_2SO_4. Autoclave or sterile filter.
10. 5052 (50×): 25% (w/v) glycerol, 2.5% (w/v) glucose, 10% (w/v) α-lactose. Autoclave or sterile filter.
11. Liquid nitrogen.

2.2 Purification of DART Protein by Affinity Chromatography

1. Qproteome Bacterial Protein Prep Kit.
2. Ni-NTA Agarose Beads.
3. Poly-Prep Chromatography column.
4. PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4.
5. Equilibration buffer: 50 mM Na_2HPO_4, 100 mM NaCl.
6. Wash buffer 1: 10 mM imidazole, 5 mM β-ME dissolved in PBS.

7. Wash buffer 2: 25 mM imidazole, 5 mM β-ME dissolved in PBS.
8. Wash buffer 3: 40 mM imidazole, 5 mM β-ME dissolved in PBS.
9. Wash buffer 4: 60 mM imidazole, 5 mM β-ME dissolved in PBS.
10. Elution buffer 1: 10 mM Tris–HCl (pH: 7.4), 100 mM NaCl, 250 mM imidazole, 5 mM β-ME.
11. Elution buffer 2: 10 mM Tris–HCl (pH: 7.4), 100 mM NaCl, 300 mM imidazole, 5 mM β-ME.
12. Elution buffer 3: 10 mM Tris–HCl (pH: 7.4), 100 mM NaCl, 500 mM imidazole, 5 mM β-ME.

2.3 Dialysis of Purified DART Protein

1. Slide-A-Lyzer Dialysis Cassette (10,000 MWCO).
2. Dialysis Buffer: 10 mM Tris–HCl (pH: 7.4), 100 mM NaCl, 1 mM DTT.
3. 18- or 21-gauge needles.
4. 5 mL syringes.

2.4 Assessing Protein Quality and Purity by Coomassie and Immunoblot

1. PVDF membranes.
2. Whatman filter paper.
3. 2× sample buffer: 50% (v/v) 4× NuPAGE™ LDS sample buffer, 20% (v/v) DTT (1 M), 30% (v/v) water. Store at −20 °C.
4. MES SDS running buffer (pH: 7.3): 50 mM MES, 50 mM Tris base, 0.1% (w/v) SDS, 1 mM EDTA.
5. NuPAGE™ 4–12%, Bis–Tris 15-well gel.
6. Transfer buffer (pH: 8.3): 25 mM Tris base, 192 mM glycine, 20% (v/v) methanol.
7. PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4.
8. PBST: PBS with 0.05% (v/v) Tween 20.
9. Blocking solution: 5% (w/v) milk in PBST. Store at 4 °C.
10. Primary antibody solution: 1:1000 dilution of Anti-HA Antibody—Rabbit monoclonal (Cell Signaling) in 5% (w/v) BSA in PBST, 0.05% (w/v) sodium azide. Store at 4 °C.
11. Secondary antibody solution: 1:10,000 dilution of HRP anti-rabbit antibody in PBST. Dilute antibody in PBST immediately before use.

2.5 Long-Term Storage of APOBEC1-YTH Purified Proteins

1. Glycerol—100%.
2. Dialysis Buffer: 10 mM Tris–HCl (pH: 7.4), 100 mM NaCl, 1 mM DTT.
3. Liquid nitrogen.

2.6 In Vitro DART Assay

1. DART buffer (10×): 100 mM Tris–HCl (pH 7.4), 500 mM KCl, 1 μM $ZnCl_2$.
2. RNaseOUT™ Recombinant Ribonuclease Inhibitor.
3. Purified APOBEC1-YTH protein.
4. RNeasy Micro Kit.
5. iScript Reverse Transcription Supermix.
6. CloneAmp™ HiFi PCR Premix.
7. 6× purple gel loading dye.
8. Agarose.
9. QIAquick Gel Extraction Kit.
10. TAE gel running buffer (50×): 40 mM Tris base, 2 mM EDTA, 20 mM glacial acetic acid.

3 Methods

3.1 Induction of DART Protein Expression in *E. coli*

1. Clone APOBEC1-YTH into the pET-His6-MBP vector so that the 6×His tag and the maltose binding protein (MBP) solubility tag are added to the N-terminal portion of the protein (*see* **Note 1**). Transform 3 μL into 50 μL of the pLysS bacterial strain.
2. Pick a colony and culture in 3 mL of LB medium with 50 μg/mL kanamycin for 16–18 h (*see* **Note 2**).
3. Make a glycerol stock by adding 500 μL of cultured cells and 500 μL of sterile 30% glycerol to a 1.5 mL tube. Mix well and quickly store at −80 °C.
4. Add a small stab from the glycerol stock to 30 mL of LB medium with 50 μg/mL kanamycin.
5. Culture 16–18 h at 37 °C with shaking (200 rpm).
6. Prepare 250 mL of autoinduction medium in a 1 L Erlenmeyer flask (*see* **Note 3**) and add 100 μg/mL kanamycin.
7. Add 6 mL of the bacterial culture to the autoinduction medium.
8. Culture for 8–10 h at 37 °C with shaking (250 rpm).
9. Culture for 16–18 h at 18 °C with shaking (250 rpm).
10. Spin cultures at >5000 × *g* for 10 min at 4 °C to pellet the bacterial cells.

11. Decant the supernatant, removing as much supernatant as possible without disturbing the pellet.
12. Carefully submerge the container with the cell pellet into liquid nitrogen for 10–15 s, or until the pellet has frozen solid.
13. The cell pellets may be stored at −80 °C at this stage. Alternatively, you may proceed directly to protein purification.

3.2 Purification of DART Protein by Affinity Chromatography

1. Thaw the cell pellets on ice or at 4 °C. It is important at this step to thaw the pellet without excessive warming.
2. Prepare the complete cell lysis buffer from the Qiagen Qproteome Bacterial Protein Prep Kit (*see* **Note 4**). Add 10 mL of complete lysis buffer and pipet up/down to resuspend the pellet in the lysis buffer. It will take some time to completely resuspend the pellet, and the solution may be quite viscous. Do not vortex to resuspend the pellet. Once resuspended, transfer to a 50 mL conical tube.
3. If multiple flasks for each sample were prepared, you can pool the lysates here.
4. Incubate on ice for 30 min. Gently swirl or shake the lysate every 5–10 min.
5. At the end of the incubation, spin the samples at 12,500 × *g* for 60 min at 4 °C to pellet the insoluble material. Carefully remove the supernatant and place in a new tube. The supernatant should be yellow in color but cleared of insoluble material. Additional spin time may be required if the lysate is cloudy. It is important to obtain a clear lysate at this step.
6. Take a 50 μL sample of the lysate for SDS-PAGE analysis (Fig. 1).
7. Carefully pipet 750 μL of resuspended Ni-NTA bead slurry into a chromatography column and allow the liquid to drain by gravity flow (*see* **Note 5**).
8. Wash the beads on the column with 6 mL of equilibration buffer. Allow the liquid to drain (*see* **Note 6**).
9. Pipet the lysate directly into the column and allow it to flow through the Ni-NTA beads. Collect the flow-through in a tube below the column. After the supernatant has drained, take a 50 μL sample of the flow-through for SDS-PAGE analysis (Fig. 1).
10. Apply 10 mL of wash buffer 1. When the buffer has finished draining, collect a 50 μL sample of the wash buffer for SDS-PAGE analysis (Fig. 1).
11. Apply 6 mL of wash buffer 2. When the buffer has finished draining, collect a 50 μL sample of the wash buffer for SDS-PAGE analysis (Fig. 1).

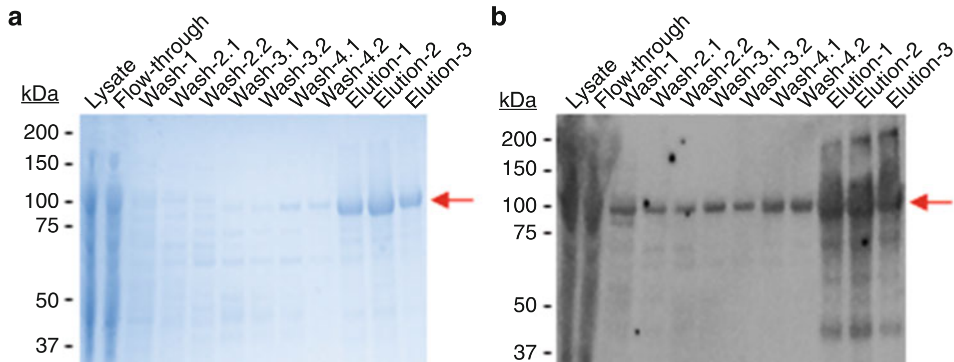

Fig. 1 SDS–PAGE analysis of APOBEC1-YTH protein purification. Red arrow labels the position of APOBEC1-YTH (96 kDa). (**a**) Coomassie blue stain of washes and elutions during the protein purification process. (**b**) Corresponding HA-tag western blot of panel (**a**)

12. Repeat **step 11** for a total of two washes.
13. Apply 6 mL of wash buffer 3. When the buffer has finished draining, collect a 50 μL sample of the wash buffer for SDS-PAGE analysis (Fig. 1).
14. Repeat **step 13** for a total of two washes.
15. Apply 6 mL of wash buffer 4. When the buffer has finished draining, collect a 50 μL sample of the wash buffer for SDS-PAGE analysis (Fig. 1).
16. Apply 10 mL of wash buffer 4. When the buffer has finished draining, collect a 50 μL sample of the wash buffer for SDS-PAGE analysis (*see* Fig. 1).
17. Apply 1.5 mL of elution buffer 1. When the buffer has finished draining, collect a 50 μL sample of the eluate for SDS-PAGE analysis (Fig. 1).
18. Apply 1.5 mL of elution buffer 2. When the buffer has finished draining, collect a 50 μL sample of the eluate for SDS-PAGE analysis (Fig. 1).
19. Apply 1.5 mL of elution buffer 3. When the buffer has finished draining, collect a 50 μL sample of the eluate for SDS-PAGE analysis (Fig. 1).
20. At this point, the eluate may be stored at 4 °C overnight (*see* **Note 7**), however it is recommended to continue with dialysis (*see* **Note 8**).

3.3 Dialysis of Purified DART Protein

1. Prepare 1 L of dialysis buffer in a 1 L beaker (*see* **Note 9**).
2. Place the Slide-a-Lyzer dialysis cassette in the beaker of dialysis buffer for 1–2 min to hydrate the membrane (*see* **Note 10**).
3. Using an 18 G or 21 G needle attached to a 5 mL syringe, aspirate the sample. Carefully insert the needle into the dialysis

cassette without touching the membrane and slowly inject the sample into the cassette. As you remove the needle, aspirate out the excess air so that there is as little remaining air as possible inside the dialysis cassette.

4. Place the dialysis cassette into the beaker of dialysis buffer. Place on a stir plate at 4 °C for 2 h at a low stir setting (*see* **Note 11**).
5. After 2 h, remove the cassette from the beaker and pour out the dialysis buffer. Add 1 L of fresh dialysis buffer and incubate for at least 2 h at 4 °C with stirring.
6. After the incubation, set a 5 mL syringe plunger at 2 mL and attach an 18 G or 21 G needle. Remove the dialysis cassette and carefully insert the needle without touching the membrane and inject 2 mL of air into the cassette. Then, invert the cassette so that the sample pools at the bottom of the cassette where the needle is inserted and draw up the plunger to completely remove all of the sample.
7. Take a 50 μL sample of the dialyzed purified protein for SDS-PAGE analysis (Fig. 1).

3.4 Assessing Protein Quality and Purity by Coomassie Stain and Immunoblot

1. Collect all 50 μL aliquots and add 50 μL of 2× sample buffer to each tube. Mix gently by pipetting (*see* **Note 12**).
2. Heat samples at ≥ 98 °C for 5 min and centrifuge briefly.
3. Load 10 μL of each sample into a well in two separate SDS-PAGE gels (*see* Fig. 1).
4. Perform electrophoresis at 150 V for ~1.5 h or until the dye reaches the bottom of the gel.
5. Remove the gels from their cassettes and rinse in 1× PBS.
6. Place one gel in 50–100 mL Coomassie stain solution for 1 h with gentle rocking at room temperature.
7. After 1 h, pour off the Coomassie stain solution and add 20 mL of Coomassie destain solution for 1 h with gentle rocking at room temperature.
8. After 1 h, pour off the Coomassie destain solution and add 20 mL of Coomassie destain solution. Cover the top of the container with Parafilm and keep on rocker at room temperature for at least 16 h.
9. Place the other gel in transfer buffer for 5 min.
10. To assemble transfer apparatus, open transfer cassette holder and place a sponge (soaked in 1× transfer buffer) on the bottom of the transfer cassette.
11. Place two pieces of filter paper (soaked in transfer buffer) on the sponge.

12. Wet the PVDF membrane in transfer buffer for 30 s and place on top of the filter papers. Use a roller to eliminate air bubbles.
13. Place the gel on the PVDF membrane and use a roller to eliminate air bubbles.
14. Place two filter papers (soaked in transfer buffer) on the gel and use a roller to eliminate air bubbles.
15. Place a sponge (soaked in transfer buffer) on the top of the assembly and close the cassette.
16. Insert the cassette into the transfer apparatus, fill the transfer tank with 1× transfer buffer, and run at 100 V for 60 min.
17. After the transfer is complete, soak the membrane in blocking solution for 1 h.
18. Place the membrane in 5 mL of primary antibody solution. Incubate with gentle rocking for at least 16 h at 4 °C.
19. Wash three times with PBST for 5 min at room temperature with rocking.
20. Place the membrane in 10 mL of secondary antibody solution. Incubate with gentle rocking for 1 h at room temperature.
21. Wash three times with PBST for 5 min at room temperature with rocking.
22. Mix 500 μL of ECL reagent A and 500 μL of ECL reagent B immediately before use.
23. Remove gel from PBST and apply 1 mL of the mixed ECL reagent to the entire membrane and incubate at room temperature for 1 min.
24. Pick up the membrane with forceps and allow the ECL reagent to drain off the membrane.
25. Immediately bring the membrane to a gel imaging system or expose using X-ray film (Fig. 1).

3.5 Long-Term Storage of APOBEC1-YTH Purified Proteins

1. Add 1.67 μg of purified protein to a clean 0.6 mL tube.
2. Add dialysis buffer for a final volume of 16 μL.
3. Add 4 μL of 100% glycerol for a final volume of 20 μL.
4. Mix thoroughly by pipetting.
5. Close tube and place in liquid nitrogen.
6. Repeat to store all of the purified protein prep.
7. Store tubes long term at −80 °C (*see* **Note 13**).

3.6 In Vitro DART Assay

1. Combine the following reagents in a 1.5 mL tube, adding the protein last (*see* **Notes 14** and **15**).
   (a) 5 μL of 10× DART buffer.
   (b) 1 μL RNaseOUT.

   (c) 50 ng total RNA.
   (d) 3 μL purified protein in 20% glycerol (250 ng).
   (e) Ultrapure water to 50 μL.
2. Gently mix by pipetting.
3. Incubate at 37 °C for 4 h (*see* **Note 16**).
4. After incubation, isolate RNA using RNeasy Micro Kit according to the manufacturer's instructions (*see* **Note 17**). Elute with 14 μL of RNase-free water.
5. The RNA can be stored at −80 °C after this step, or cDNA synthesis can be performed immediately following cleanup. The following steps describe targeted PCR amplification of a specific RNA region followed by Sanger sequencing to observe editing at specific loci. However, the purified RNA may also be used to prepare next-generation sequencing libraries for RNA-seq (*see* **Note 18**).
6. Add 7 μL of purified RNA to a clean PCR tube.
7. Add 9 μL of ultrapure water.
8. Add 4 μL of 5× iScript Supermix for RT (*see* **Note 19**).
9. Mix gently by pipetting and run the following program on a thermocycler:
   (a) 5 min at 25 °C.
   (b) 20 min at 46 °C.
   (c) 1 min at 95 °C.
   (d) Hold at 4 °C.
10. Dilute cDNA 1:5 with ultrapure water.
11. To generate an amplicon for Sanger sequencing, assemble the following reagents in a clean PCR tube (*see* **Note 20**):
   (a) 10 μL CloneAmp 2× mastermix.
   (b) 1 μL forward primer.
   (c) 1 μL reverse primer.
   (d) 1 μL diluted cDNA.
   (e) 7 μL ultrapure water.
12. Mix gently by pipetting and run the following program on a thermocycler:
   (a) 98 °C for 45 s.
   (b) 98 °C for 10 s.
   (c) 57 °C for 10 s.
   (d) 72 °C for 5 s.
   (e) Repeat **steps b–d** for a total of 30 cycles.

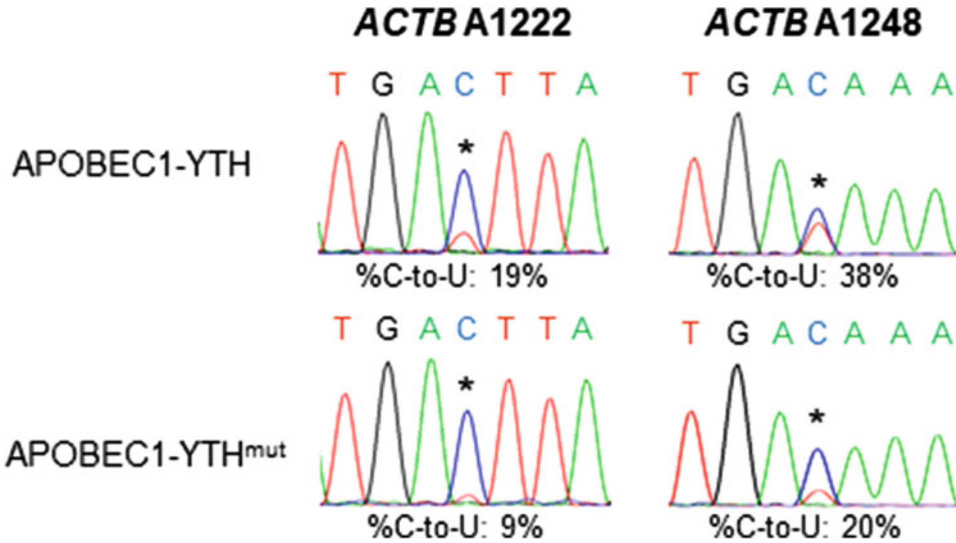

Fig. 2 In vitro DART was performed on 50 ng of total RNA from HEK293T cells, followed by RT-PCR to amplify the *ACTB* 3′UTR. Sanger sequencing chromatograms show C-to-U mutations in cytidine residues adjacent to two m^{6}A sites in *ACTB*: A1222 (left) and A1248 (right). C-to-U mutation rate is indicated for each site and indicates decreased editing using APOBEC1-YTH^{mut} protein compared to APOBEC1-YTH

(f) 72 °C for 5 min.

(g) Hold at 4 °C.

13. Add 4 μL of 6× purple gel loading dye and mix by pipetting.
14. Load samples into a 1% agarose gel and run until ladder is sufficiently separated.
15. Use a razorblade to excise desired products from the gel.
16. Extract DNA using Qiagen QIAquick Gel Extraction Kit according to manufacturer's protocol.
17. Perform Sanger sequencing using a primer of interest. We typically use one of the primers used for PCR in **step 11** above (Fig. 2).
18. Assess C-to-T transitions in Sanger sequencing data. We use EditR [6] to quantify % C-to-T (*see* **Note 21**).

4 Notes

1. Detailed cloning instructions can be found on the Addgene page for plasmid #29656.
2. Prepare all solutions using Milli-Q water. Prepare and store all reagents at room temperature (unless specified otherwise). Follow all chemical safety guidelines when preparing solutions. Follow all waste disposal regulations when disposing of waste materials.

3. Using beveled flasks can increase yield.
4. Ensure that the lysozyme in the lysis buffer is completely dissolved. This may require time and gentle warming followed by cooling the buffer again.
5. Keep all reagents on ice during protein purification or work in a cold room. Further, we use the Ni-NTA resin from GoldBio most successfully under the conditions used in this protocol. Substituting different resins with different binding capacities may require optimization to maximize yield and purity.
6. Do not allow the liquid to completely drain and the beads to dry. Always add the next wash/buffer right as the liquid level reaches the top of the beads.
7. Do not mix the elutions until you have confirmed their quality.
8. It is recommended to proceed with dialysis immediately after purification as there is a high concentration of imidazole in the elution buffers, and the protein is only stable for several weeks when stored at 4 °C.
9. Always make fresh dialysis buffer as it contains DTT.
10. Never touch the membrane of the dialysis cassette.
11. Each dialysis step should proceed for at least 2 h to allow for complete buffer exchange. However, the steps may proceed for 16–18 h if needed.
12. The Coomassie stained gel is for the assessment of purity, while the immunoblot is performed to confirm the expected band is indeed APOBEC1-YTH (this fusion protein contains an HA tag). It is highly recommended to perform this quality control step, as it may help with troubleshooting if a prep is unsuccessful.
13. Protein is still active when stored at −80 °C for more than 9 months.
14. It is highly recommended that RNA used for in vitro DART assays is first treated with DNase I, followed by ethanol precipitation.
15. Less than 50 ng of RNA may be used, if needed. In vitro DART assays using as little as 30 ng per reaction have been performed successfully in our hands as described. If using significantly more or less RNA, optimization of the RNA–protein ratio is recommended. For example, if using significantly more than 50 ng of RNA it is recommended to increase the protein concentration and/or time of incubation. If using less RNA, it is recommended to decrease the protein concentration and/or time of incubation.
16. Shaking during the 4-h incubation does not seem to increase or decrease editing efficiency.

17. Phenol–chloroform extraction or TRIzol extraction may also be used to isolate RNA after the DART assay. However, the RNeasy Micro Kit is recommended for its ease of use.
18. To make next-generation sequencing libraries, particularly if less than 50 ng of input RNA is used, it is recommended to use a template-switching approach, such as the Low Input/Single Cell Library Prep Kit from New England Biolabs.
19. Any cDNA synthesis system that can work with low input RNA can be used (e.g., SuperScript III).
20. Any high-fidelity polymerase can be used for amplicon generation. The annealing temperature and extension time should be adjusted for each primer pair and amplicon.
21. An alternate method of quantifying C-to-U editing is to use ImageJ to calculate the height of the C and T peak at the edited site and calculate % C-to-T = $T_{height}/(T_{height} + C_{height})$. This method does not consider the background in sequencing traces, nor the shape of the peaks, and is therefore not recommended.

References

1. Flamand MN, Meyer KD (2019) The epitranscriptome and synaptic plasticity. Curr Opin Neurobiol 59:41–48
2. Meyer KD, Jaffrey SR (2017) Rethinking m ^{6} a readers, writers, and erasers. Annu Rev Cell Dev Biol 33(1):319–342
3. Zaccara S, Ries RJ, Jaffrey SR (2019) Reading, writing and erasing mRNA methylation. Nat Rev Mol Cell Biol 20:608–624
4. Roundtree IA, Evans ME, Pan T, He C (2017) Dynamic RNA modifications in gene expression regulation. Cell 169(7):1187–1200
5. Meyer KD (2019) DART-seq: an antibody-free method for global m6A detection. Nat Methods 16:1275–1280
6. Kluesner MG, Nedveck DA, Lahr WS et al (2018) EditR: a method to quantify base editing from sanger sequencing. CRISPR J 1 (3):239–250

Chapter 21

Target-Specific Profiling of RNA m⁵C Methylation Level Using Amplicon Sequencing

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Abstract

Mapping the position and quantifying the level of 5-methylcytosine (m^5C) as a modification in different types of cellular RNA is an important objective in the field of epitranscriptomics. Bisulfite conversion has long been the gold standard for the detection of m^5C in DNA, but it can also be applied to RNA. Here, we detail methods for bisulfite treatment of RNA, locus-specific PCR amplification, and detection of candidate sites by sequencing on the Illumina MiSeq platform.

Key words 5-methylcytosine, Epitranscriptomics, Bisulfite conversion, Next-generation sequencing, MiSeq

1 Introduction

Cellular RNAs can be richly modified with more than one hundred known chemically and structurally distinct nucleoside modifications [1–3]. The field of epitranscriptomics [4–6] has been greatly accelerated by the development of high-throughput mapping methods for RNA modifications, typically based on a next-generation sequencing (NGS) readout. Transcriptome-wide positions of 5-methylcytosine (m^5C) [7, 8], 5-hydroxymethylcytosine [9, 10], N^6-methyladenosine [11–13], N1-methyladenosine [14–16], and pseudouridine [17–19] have each been reported in this way. To detect m^5C in RNA, a range of methods have been developed, including the direct (meRIP [20]) or indirect immunoprecipitation of methylated RNA (aza-IP [21], miCLIP [22]). The bisulfite conversion approach in popular use for DNA methylation detection has also been successfully adapted to RNA [8, 23–

Tennille Sibbritt and Ulrike Schumann contributed equally to this work.

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