Detection technologies for RNA modifications

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To date, more than 170 chemical modifications have been characterized in RNA, providing a new layer of gene expression regulation termed the ‘epitranscriptome’. RNA modification detection methods and tools advance the functional studies of the epitranscriptome. According to the detection throughput and principles, existing RNA modification detection technologies can be categorized into four classes, including quantification methods, locus-specific detection methods, next-generation sequencing-based detection technologies and nanopore direct RNA sequencing-based technologies. In this review, we summarize the current knowledge about these RNA modification detection technologies and discuss the challenges for the existing detection tools, providing information for a comprehensive understanding of the epitranscriptome.

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INTRODUCTION
RNA is a single-stranded molecule consisting of four nucleotides: adenosine (A), guanosine (G), cytidine (C) and uridine (U). RNA, both an important and conserved macromolecule, not only participates in the flow of genetic information but also regulates gene expression. Beyond sequence information, chemical modifications add to the complexity of RNA, emerging as a new layer of gene expression regulation. Since the first chemical modification was characterized 60 years ago, more than 170 RNA modifications have been characterized. Most of the RNA modifications have been identified in abundant non-coding RNAs, including ribosomal RNA (rRNA), transfer RNA (tRNA), and small nuclear RNA (snRNA).

The development of detection technologies advances the investigation of the functional roles of RNA modifications. To date, more than ten chemical modifications have been mapped in a transcriptome-wide manner, including N⁶-methyladenosine (m⁶A), N⁷, 2′-O-dimethyladenosine (m⁷Am), 5-methylcytosine (m⁵C), 5-hydroxymethylcytosine (hm⁵C), inosine (I), pseudouridine (Ψ), N²-methyladenosine (m²A), 2′-O-methylthionine (Nm), N²-acetylcytidine (ac⁴C), N⁷-methylguanosine (m⁷G) and dihydrouridine (D). Different chemical modifications play distinct regulatory roles in RNA metabolism and function. For instance, m⁶A, the most abundant internal messenger RNA (mRNA) modification, influences RNA metabolism in multiple ways, including stability, splicing, translation, localization and RNA secondary structure. m⁷G in mRNA increases mRNA translation efficiency and augments miRNA biogenesis. ac⁴C in mRNA promotes translation, and ac⁴C in rRNA can affect rRNA biogenesis.

RNA modification detection technologies provide not only resources for a comprehensive understanding of the epitranscriptome but also tools for functional studies. Hence, in this review, we will summarize the current knowledge about these existing RNA modification detection technologies and discuss the challenges for these existing detection tools. The detection technologies are categorized according to the detection throughput and principles into four classes: quantification methods, locus-specific detection methods, next-generation sequencing-based detection technologies and nanopore direct RNA sequencing-based technologies.

RNA MODIFICATION QUANTIFICATION METHODS
The identification and quantification of new modified nucleotides requires powerful RNA modification quantification methods. Based on the principle that modified nucleotides possess distinct chemical properties from the originals, several RNA modification quantification methods have been established, including two-dimensional thin-layer chromatography (2D-TLC), dot blot, and liquid chromatography–mass spectrometry (LC–MS). These approaches can be used to quantify the modification abundance in specific RNA species and require highly purified RNA due to the lack of sequence information.

2D-TLC
2D-TLC is a widely used RNA modification detection method according to the distinct mobilities of different nucleotides in the solvent. In detail, isolated RNA is first partially digested into oligonucleotides using RNase A, T1, or T2 and then labeled with ³²P...
using T4 polynucleotide kinase (T4 PNK). Finally, $5'\text{-}32\text{P}-\text{NMP}$ is acquired by nuclease P1 digestion and further separated by 2D-TLC. The nucleotides can be determined by assignment to the standards by comparing their retardation factor (Rf) values (Fig. 1). Quantification of the nucleotides is achieved by measuring the radioactivity of the corresponding spots in the TLC plate. This approach is very sensitive and requires only a small amount of RNA (from 50 ng to 200 ng). Consequently, this approach can be applied to both abundant non-coding RNAs (rRNA and tRNA) and less abundant mRNA. In addition, it can also detect RNA modifications in specific tRNA or snRNA sequences, which can be isolated by gel purification or hybridization methods. Furthermore, this approach does not require expensive instruments and thus can be run inexpensively. However, this method also has certain drawbacks, including the requirement of a radioactive reagent, the bias caused by RNase digestion and discrepant $^{32}\text{P}$ labeling efficiency for the modified nucleotides.

**Dot blot**

Dot blot assay is established and applied to detect and quantify modification levels in RNAs using a specific antibody. In detail, isolated RNAs are stalled to the polyvinylidene fluoride (PVDF) or nitrocellulose membrane directly without electrophoretic size separation. A specific antibody for target modification is incubated with the membrane, followed by secondary antibody hybridization and subsequent signal trapping (Fig. 1). Comparing results from different experimental groups can obtain semiquantitative modification level information. This approach has been widely applied to various RNA species, including non-coding RNAs and mRNAs. Given the detection principle, the sensitivity and accuracy of this approach are highly dependent on the specificity of the antibody. In addition, the amount of starting material, ranging from nanograms to micrograms, also depends on the abundance of the RNA modifications of interest. Since the workflow is straightforward and can be performed inexpensively, this approach has been widely applied for RNA modification detection. However, the lack of absolute quantification and locus information limits a wider application of this approach.

**HPLC and LC–MS**

High-performance liquid chromatography (HPLC) is an advanced column chromatography technology to separate nucleosides according to their distinct polarities. Prior to HPLC analysis, RNA or oligonucleotides are digested and dephosphorylated to single nucleosides by nuclease P1 and alkaline phosphatase. The UV absorbance and retention time of the nucleosides are recorded by a UV detector, which can be used to identify and measure the abundance of the modified nucleosides (Fig. 1). Compared with 2D-TLC, HPLC analysis is rapid and free of radiolabeling. However, this approach can only be applied to detect highly abundant modifications in abundant RNA species, such as rRNA, tRNA and synthetic RNAs, and requires a large amount of purified RNA (more than 1 µg) due to the detection limit of the UV detector.

To increase detection sensitivity, HPLC is coupled with mass spectrometry. Similarly, RNA or oligonucleotides are completely digested to nucleosides and separated by reverse column chromatography. Then, these nucleosides are ionized and further fragmented into specific product ions via mass spectrometry. Integration of retention time, mass-to-charge ratio (m/z) and product ion are capable of determining a certain nucleoside (Fig. 1). In addition, quantification of nucleosides can be achieved through the external standard curve in the same batch. The extremely high sensitivity of triple quadrupole-based mass spectrometry provides this approach a detection limit that can reach the low femtomolar range, and the amount of starting material can be as low as 50 ng, thereby allowing the determination and quantification of these low-abundance modifications in mRNA and low-abundance ncRNA. Hence, LC–MS has been a benchmark for RNA modification detection and quantification. However, the limit of this technique is the requirement of instruments including HPLC and mass spectrometry. In addition, due to the lack of sequence information, when detecting and quantifying RNA modifications in mRNA or other less abundant
RNA types, caution must be taken to reduce contamination from abundant and highly modified sRNA and tRNA.

**LOCUS-SPECIFIC DETECTION METHODS**

The precise position information of a certain RNA modification is important for functional studies. To date, several locus-specific RNA modification detection methods have been developed. These methods can be categorized according to the detection principles into four classes: (1) Primer extension; (2) RNase H-based approaches; (3) electrospray ionization-mass spectrometry (ESI-MS)-based approaches; and (4) semiquantitative PCR- or qPCR-based approaches.

**Primer extension**

This approach is based on reverse transcription and has been used extensively to detect and localize various RNA modifications, including m1A, Ψ, and m1G. In principle, a 5′-labeled specific RT primer is hybridized with the RNA of interest and extended by reverse transcriptase. Hence, this approach relies on prior knowledge of the modification type and sequence information of the target RNA. Without modifications, reverse transcriptase can reach the 5′ end of RNA and generate full-length cDNA. When encountering modified nucleotides, the extension of reverse transcriptase is blocked immediately upstream of the modified site. Then, the RT products are separated using denaturing polyacrylamide gels, and the terminal position of truncated cDNA indicates the modified nucleotide position. The truncated and full-length cDNA are then subjected to end-labeling followed by ligation-assisted extraction and thin-layer chromatography, with the labeled nucleotides being sequenced to identify the exact position of the modified nucleotide.

**RNase H-based approach**

The RNase H-based approach is independent of reverse transcription and thus can detect and quantify RNA modifications that do not affect Watson-Crick base pairing. In this method, purified RNA is cleaved into two halves at the 5′ end of the nucleotide of interest by RNase H, and cleavage specificity is achieved by annealing with a specific 2′-O-methyl RNA–DNA chimera oligonucleotide. Then, the 3′-half of the RNA is purified, and its 5′ terminus is further labeled with 32P. Furthermore, the oligonucleotides are completely digested into single nucleotides and subsequently resolved by TLC. Owing to the radiolabeling specificity, only the modified and unmodified forms of the nucleotide of interest can be detected and accurately quantified by TLC analysis.

**Semiquantitative PCR or qPCR-based approach**

Similar to primer extension assays, semiquantitative PCR or qPCR-based approaches are also based on the fact that modified nucleotides impede reverse transcriptase extension. In contrast to primer extension and RNase H-based approaches, qPCR-based approaches are free of radiolabeling and hence are time-saving and easy to perform in the laboratory. To date, several semiquantitative PCR- or qPCR-based approaches have been developed and have successfully detected Nm, Ψ, and m1A in diverse RNA species. In addition, owing to the high sensitivity and specificity of PCR and qPCR, these approaches do not require purified RNA as the starting material and can be applied to various RNA species, including less abundant mRNA and lncRNA.

Nm blocks reverse transcriptase extension at a low dNTP concentration and allows readthrough at a high dNTP concentration. Taking advantage of this property, researchers have developed a method referred to as RTL-P (reverse transcription at low dNTP concentrations followed by PCR). In this method, the RNA of interest is first reverse-transcribed at both low and high dNTP concentrations. The truncated and full-length cDNA are amplified by specific primers. The PCR products are analyzed by gel electrophoresis, and the Nm status can be determined by comparing the intensity of the longer PCR product with that of the shorter product. In addition to altering RT conditions, RT enzymes can also be engineered to facilitate modified nucleotide detection. For instance, an engineered thermostable KlenTaq DNA polymerase variant possesses reverse transcription activity and can discriminate Nm at normal dNTP concentrations. The combination of this engineered DNA polymerase with qPCR has achieved expeditious quantification of Nm.

In addition to the engineered thermostable KlenTaq DNA polymerase variant, two other DNA polymerases, Tth and Bst, also have reverse transcriptase activity and exhibit distinct capacities to extend when encountering m1A residues versus A residues, which allows locus-specific detection of m1A.

Moreover, to increase the detection sensitivity of m1A, another method referred to as the single-base elongation- and ligation-based PCR amplification method (SELECT) has been developed. Because m1A can both hinder the elongation activity of DNA polymerases and reduce the nick ligation efficiency of SplintR ligase, in SELECT, cDNA products formed from m1A-containing RNA templates are dramatically reduced, hence significantly increasing detection robustness. Based on qPCR, SELECT is a powerful tool to quantify the m1A fraction in a linear manner.

In addition to truncations, the property of induced mutation/deletions by modified nucleotides can also be utilized for locus-specific detection. For instance, Ψ can be selectively labeled by N-cyclohexyl-N′-(2-morpholinoethyl) carbodiimide (CMC), and the Ψ-CMC adducts interfere with Watson-Crick base pairing during RNase T1/A digestion. The splint ligation enables RNA mixtures to be used as the starting material rather than a specific purified RNA, and modifications in mRNA and lncRNA can thus also be quantified. The accurate size of the DNA oligonucleotide is recovered by gel purification. Then, the 32P-labeled nucleotides are released by nuclease P1 digestion and further analyzed by TLC (Fig. 2b). This approach has been applied to quantify m1A and Ψ modification status in mRNA. Since this approach avoids reverse transcription, it can be used as an orthogonal method to validate modification sites identified by transcriptome-wide methods that are dependent on reverse transcription. As with other RNase H-based methods, SCARLET also requires prior knowledge of the sequence information to target a single specific nucleotide and cannot quantify the modification status in a de novo manner.

In addition, the requirement of radioactive reagents and complicated procedures limit wider application of this method.

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reverse transcription. Under optimized RT conditions, Ψ–CMC adducts can be read through and induce mutation/deletion in cDNA. Such mutation/deletions alter the melting curves of the qPCR products, thus enabling the locus-specific detection of Ψ modification (Fig. 2e)\textsuperscript{95}.

**ESI-MS-based approach**

In contrast to the LC–MS-based nucleoside quantification strategy described above, in this approach, isolated RNA samples are first digested into 5–15 nucleotide fragments by selective endoribonucleases, such as RNase T1, RNase A, and RNase U2. Then, the
oligonucleotides are separated by HPLC, and the sequence ladders from the oligonucleotides are further generated through ESI-MS, which can be used for sequence reconstruction and modification identification (Fig. 2f). Hence, this approach can provide both site and stoichiometry information for the modifications of interest. Compared with other locus-specific methods, the ESI-MS-based approach does not rely on prior knowledge of sequence information and thus is able to detect and quantify RNA modification in a de novo manner. Therefore, this approach has been widely applied for modification detection in abundant RNAs, including tRNA, rRNA, and snRNA. Recently, this approach has been used to detect modifications in miRNA and cap modifications in mRNA. The major limitation of this approach is the requirement for highly sensitive ESI-MS. In addition, given the detection principle, the requirement of starting material is large; thus, this method is only suitable for abundant RNA species.

**NEXT-GENERATION SEQUENCING-BASED DETECTION TECHNOLOGIES**

With the benefit of advances in next-generation sequencing, an increasing number of RNA modification sequencing technologies have been developed. Such technologies represent powerful tools to map modified nucleotides in a transcriptome-wide manner and promote the elucidation of the regulatory roles of RNA modifications. Chemical modifications alter the inherent features of the original nucleotides, including base-pairing performance in reverse transcription, chemical reaction activities, enzymatic reaction activities and binding affinities with specific proteins or antibodies. Therefore, coupled with next-generation sequencing, these properties of modified nucleotides can be used to characterize RNA modifications throughout the transcriptome. The existing sequencing technologies can be categorized according to the detection principles into four classes: (1) direct sequencing technology, (2) chemical-assisted sequencing technology, (3) antibody-based sequencing technology, and (4) enzyme/protein-assisted sequencing technology (Table 1).

**Direct sequencing technology**

For some modified nucleotides, the existence of chemical modifications alters canonical Watson-Crick base pairing during reverse transcription and further leads to truncation or misincorporation in cDNA synthesis. Therefore, this feature can be used to map modified nucleotides throughout the transcriptome. For instance, in contrast to adenosine, inosine pairs with cytidine in reverse transcription, and thus, the A-to-I editing position can be identified by comparing the genomic DNA and RNA sequencing data and detecting A-to-G mismatch sites (Fig. 3a). Although this approach is widely applied to A-to-I editing site detection, caution is still needed to reduce the false positives introduced by SNPs, somatic mutations, pseudogenes and sequencing errors.

Aside from inosine, several other chemical modifications at the Watson-Crick face of the nucleobase can also induce RT stops or mutations in cDNA synthesis, thereby allowing base-resolution detection.

In contrast to inosine, the reverse transcription signatures of these modifications are not constant and dependent on both the surrounding sequences and the RT conditions. Furthermore, to increase the detection accuracy, several improvements have been made. For instance, the *E. coli* AlkB demethylase and its mutant treatment are introduced to remove m1A, m3C, m1G, N2,N2’-dimethylguanosine (m2’G), and 3-methyluridine (m3U) in RNA prior to cDNA synthesis, and high-confidence methylation sites in the tRNA transcriptome can thus be identified by comparing the data for parallel sequenced, demethylase-treated and untreated samples (Fig. 3b). Hence, to improve detection sensitivity, an HIV-1 reverse transcriptase against m1A was developed that allows m1A detection in human mRNA. Moreover, RT systems, including enzymes and reaction conditions, can be optimized to detect modified nucleotides that do not interfere with Watson-Crick base pairing. For instance, Nm, an RT-silent modification, can block cDNA synthesis when the concentration of dNTPs or Mg2+ in RT reactions is limited. Coupling this feature with next-generation sequencing, two methods, 20ME-seq and MeTH-seq, have achieved a transcriptomic profile of Nm at base resolution (Fig. 3b). As mentioned above, some DNA polymerases also possess reverse transcriptase activity and show differential elongation ability when encountering m3A residues versus A residues. To advance this feature, the KlenTaq DNA polymerase was evolved and the evolved variant exhibited significantly increased error rates opposite m3A but not unmodified A, enabling direct identification of m3A by analyzing the mutational signal from sequencing data (Fig. 3b). In addition, substitution of 4SedTTP (atom-specific replacement of oxygen with selenium at the 4-position) for dTTP under RT conditions can also facilitate m3A detection. In principle, compared with 4SeET-A, 4SeET-m3A pairing is unfavorable and results in aborted cDNA synthesis opposite m3A sites, thereby allowing for the discrimination of m3A from A. Furthermore, with the assistance of the m3A demethylase FTO through high-throughput sequencing, m3A can be precisely identified within the mammalian transcriptome at single-nucleotide resolution.

**Chemical-assisted sequencing technologies**

Chemical treatments are widely exploited to discriminate modified nucleotides from unmodified nucleotides in three ways: (1) installing biotin tags to enrich modified transcripts; (2) altering the base-pairing features to induce misincorporation or truncation in reverse transcription; and (3) chemical-induced cleavage followed by specific adaptor ligation (Fig. 4). The enrichment strategy facilitates RNA modification detection in low-abundance RNA species and low-stoichiometry modifications. Some modified nucleotides can be labeled by specific chemical reactions and further conjugated with the biotin molecules, thus enabling streptavidin enrichment. For instance, in borohydride reduction sequencing (BoRed-seq), total RNA is treated with NaBH4 and subsequently exposed to low pH to generate abasic sites at m7G positions, which can be tagged with biotin molecules for further streptavidin pulldown (Fig. 4a). Direct m3A labeling is...
Table 1. Features of next-generation sequencing-based detection technologies.

| Strategy                          | Method                        | Modification type | Detection principle                                                                 | RNA species                        | Reference  |
|----------------------------------|-------------------------------|-------------------|-------------------------------------------------------------------------------------|-----------------------------------|------------|
| Direct sequencing                | RNA and DNA differences (RDD)| Inosine           | Comparison of the genomic DNA and RNA sequencing data and detection of A-to-G mismatch sites | total RNA/mRNA/poly(A)-RNA/small RNA (18–30 nt) | 111–114    |
| ARM-seq/DM-trRNA-seq             | m^1^A, m^3^C, m^3^G, m^2^G, and m^3^U |                   | Detection of mutation signals induced by methylations at the Watson-Crick face and samples treated by WT/mutant AlkB demethylase are used as negative controls to reduce false-positive sites | small RNA (<200 nt)/rRNA/trRNA     | 117–120    |
| m1A-quant-seq                    | m^1^A                         |                   | Evolution of the HIV-1 reverse transcriptase to allow sensitive m^1^A detection in low-abundance RNA and low-stoichiometry sites | mRNA                              | 122        |
| 2OMe-seq/MeTH-seq                | Nm                            |                   | Limiting the concentration of dNTP or Mg^{2+} in RT reactions causes RT stops at Nm sites | rRNA/mRNA                          | 123,124    |
| Direct m^6^A Sequencing          | m^6^A                         |                   | Evolution of KlenTaq DNA polymerase as the RTase and induces RT misincorporations at m^6^A sites | model sequence trRNA               | 125        |
| 4SedTTP-involved and FTO-assisted sequencing | m^9^A                 |                   | Substitution of dTTP with 4SedTTP under RT conditions, which induces RT stops at m^9^A sites; FTO-treated samples are used as negative controls | model sequence                     | 126        |
| Chemical-assisted sequencing     | BoRed-seq                     | m^7^G             | m^7^G methylation is treated to generate abasic sites and further tagged with biotin molecules, enabling enrichment by streptavidin pulldown | mRNA                              | 53         |
| m6A-SEAL-Seq                     | m^6^A                         |                   | m^6^A is first oxidized to hm^6^A by FTO and further converted to dm^6^A through thiol addition; dm^6^A can be conjugated to biotin molecules, thereby enabling streptavidin pulldown | mRNA                              | 127        |
| ICE-seq                          | Inosine                       |                   | Inosine can be selectively labeled with acrylonitrile, and the resulting ce^1^ induces RT stops | mRNA                              | 128        |
| RNA-BisSeq/BS-seq                | m^3^C                         |                   | Compared with unmodified C, m^3^C is resistant to bisulfite treatment and thus can be characterized by detecting nonconverted Cs | mRNA/total RNA/large RNA (>200 nt)/small RNA (<200 nt) | 16,129–133 |
| Ψ-Seq/Pseudo-seq/PSI-seq         | Ψ                             |                   | Ψ can be labeled by CMC, and the resulting CMC-Ψ adducts induce RT stops | mRNA/total RNA/trRNA              | 134–136    |
| CeU-seq                          | Ψ                             |                   | Ψ is labeled with N^δ^CMC and further conjugated with biotin, thereby enabling preenrichment of Ψ-containing RNA and further base-resolution detection of low-stoichiometry Ψ sites | mRNA                              | 86         |
| RBS-seq                          | m^3^C, m^1^A, Ψ               |                   | Optimization of bisulfite treatment conditions and simultaneous detection of all three modifications in the same RNA | mRNA/rRNA/trRNA                   | 131        |
| m^7^G-MaP-seq                    | m^7^G                         |                   | m^7^G residues can be converted to abasic sites upon NaBH_4 treatment and further recorded as misincorporations through reverse transcription | rRNA/trRNA                        | 137        |
| m^7^G-seq                        | m^7^G                         |                   | m^7^G residues are reduced to abasic sites and further generate biotinylated AP sites, which can be enriched by biotin pulldown and induce RT stops when using HIV reverse transcriptase | mRNA/rRNA/trRNA                   | 54         |
| Rho-seq                          | D                             |                   | D can be reduced by NaBH_4 treatment and further labeled by Rho, which can induce RT stops in cDNA synthesis | mRNA/trRNA                        | 138        |
| Strategy | Method | Modification type | Detection principle | RNA species | Reference |
|----------|--------|-------------------|---------------------|-------------|-----------|
| ac4C-seq | ac\(^4\)C | ac\(^4\)C can be reacted with NaCNBH\(_3\) under acidic conditions to form N4-acetyltetrahydrocytidine, which induces misincorporation during cDNA synthesis | total RNA/mRNA | 139 |
| m\(^6\)A-label-seq | m\(^6\)A | m\(^6\)A is converted to \(\overset{\alpha}{m}\)A by metabolic labeling with S-allyl-L-selenohomocysteine, and \(\overset{\alpha}{m}\)A RNA can be further converted to cyc-A, which can induce misincorporations during cDNA synthesis | mRNA | 140 |
| m6A-SAC-seq | m\(^6\)A | m\(^6\)A is converted to \(\overset{\alpha}{m}\)m\(^6\)A by specific dimethyltransferases, and cyclized \(\overset{\alpha}{m}\)m\(^6\)A can induce misincorporation during cDNA synthesis | mRNA/rRNA-depleted RNA | 141 |
| RiboMeth-seq | Nm | Nm is resistant to alkaline hydrolysis; therefore, it can be mapped by analyzing read-end information in sequencing data | rRNA | 142–144 |
| RibOxi-seq/Nm-seq | Nm | Treatment with iterative OED cycles to remove unmodified nucleotides and selective ligation of Nm-modified ends | mRNA/rRNA | 145,146 |
| AlkAniline-Seq/TRAC-seq | m\(^3\)C, m\(^7\)G | m\(^3\)C and m\(^7\)G is resistant to NaBH\(_4\)-aniline treatment and cleavage, thereby enabling selective ligation to enrich modified fragments | rRNA/trNA | 147,148 |
| HAC-seq | m\(^3\)C | m\(^3\)C-modified sites can be specifically cleaved upon hydrazine/aniline treatment and can therefore be mapped by calculating the cleavage ratio | rRNA/RNA depleted tRNA/total RNA | 149 |
| HydraPsiSeq | \(\Psi\) | \(\Psi\) modified sites are resistant to hydrazine/aniline-induced cleavage, and the protection signal can be identified by comparing the cleavage efficiency of neighboring unmodified U sites | rRNA/mRNA | 150 |

**Antibody-based sequencing**

| Strategy | Method | Modification type | Detection principle | RNA species | Reference |
|----------|--------|-------------------|---------------------|-------------|-----------|
| m\(^5\)A-seq/MeRIP | m\(^5\)A/m\(^5\)Am | Certain modification-containing RNA fragments can be enriched by specific antibody immunoprecipitation | total RNA/mRNA | 151,152 |
| m\(^1\)A-ID-seq/m\(^1\)A-seq | m\(^1\)A | | mRNA | 153 |
| hMeRIP | hm\(^3\)C | | mRNA | 154 |
| acRIP-seq | ac\(^4\)C | | mRNA | 155 |
| m7G-RIP-Seq/m7G-Seq/m\(^7\)G MeRIP | m\(^7\)G | | mRNA/ small RNA (<200 nt)/miRNA | 156,157,148 |
| m\(^6\)A-PA6-Seq/PA-m\(^6\)A-seq | m\(^6\)A | Introducing UV-induced RNA-antibody crosslinking around modified sites, thereby inducing truncations or misincorporations during cDNA synthesis | mRNA | 158,159 |
| m\(^7\)G miCLIP-seq | m\(^7\)G | | mRNA | 155,156 |
| m\(^1\)A-MAP/m\(^1\)A-seq/m\(^1\)A-IP-Seq | m\(^1\)A | Coupling m\(^1\)A immunoprecipitation with enzyme/chemical treatment and taking advantage of m\(^1\)A-induced mutational RT signatures to achieve single-base resolution detection | mRNA | 42,122,158 |
| m\(^5\)Am-seq | m\(^5\)Am | Selective in vitro demethylation for m\(^5\)Am to discriminate m\(^5\)Am from m\(^5\)A | >200 nt RNA | 159 |

**Enzyme/protein-assisted sequencing**

| Strategy | Method | Modification type | Detection principle | RNA species | Reference |
|----------|--------|-------------------|---------------------|-------------|-----------|
| AZA-IP | m\(^5\)C | Metabolic labeling with 5-aza-C to form a covalent bond with m\(^5\)C methyltransferase, thereby enabling immunoprecipitation of the direct targets of m\(^5\)C methyltransferases | total RNA | 160 |
| m\(^5\)C-miCLIP | m\(^5\)C | Overexpression of mutated Nsun2, which can form covalent bonds with its target sites | total RNA | 161 |
| Strategy | Method | Modification type | Detection principle | RNA species |
|----------|--------|-------------------|---------------------|-------------|
| DART-seq | m^6A | Fusion of APOBEC1 to the m^6A-binding YTH domain, total RNA | m^6A is first oxidized to hm^6A by the demethylase FTO, and hm^6A is further converted to N^6-dithiolsitolmethyladenosine (dm^6A) through DTT-mediated thiol addition. The free sulphydryl group of dm^6A enables biotin to be conjugated to the m^6A-modified transcripts, thereby facilitating streptavidin enrichment and sequencing (Fig. 4a) | total RNA |
| scDART-seq | m^6A | Integration of DART-seq with a single-cell RNA-sequencing platform to achieve profiling of the m^6A methylome in single cells | + mRNA |
| EndoVIPER-seq | Inosine | In the presence of Ca^2+, E. coli Endonuclease V prefers binding to inosine in RNA, which enables high-affinity capture of inosine | mRNA |
| m^6A–REF-seq/MAZTER-seq | m^6A | MazF can specifically cleave the unmethylated 5’-ACA-3’ motif to distinguish m^6A from A | mRNA |
| SEAL-Seq | m^6A | FTO-assisted m^6A selective chemical labeling conditions, and the formed reduced nucleobase, N-biotin through a click reaction. Hence, the CMC. The presence of an azido group enables conjugation with dithiolsitolmethyladenosine (dm^6A) through DTT-mediated thiol addition. The free sulphydryl group of dm^6A enables biotin to be conjugated to the m^6A-modified transcripts, thereby facilitating streptavidin enrichment and sequencing (Fig. 4a) | mRNA |

The detection strategy of altering the base-pairing properties of nucleotides by chemical treatment has been widely applied to the transcriptome-wide detection of various modifications, including inosine, m^7C, Ψ, m^6G, ac^C, D and m^6A. For inosine detection, the direct sequencing approach is disturbed by background noise. To overcome this limitation, a selective chemical labeling reaction with acrylonitrile is adopted, and the formed N^-cyanoethylinosine (ce1I) blocks reverse transcription, resulting in truncation of cDNA. Coupling this chemical reaction with sequencing, referred to as inosine chemical erasing sequencing (ICE-seq), can achieve base-resolution inosine detection throughout the transcriptome (Fig. 4a)\textsuperscript{128}. Bisulfite treatment selectively converts unmethylated cytidine into uridine, leading to a C-to-T transition in the sequencing data; thus, transcriptome-wide m^5C can be characterized by detecting nonconverted Cs in the sequencing data (Fig. 4a)\textsuperscript{129,130}. Furthermore, to reduce false positives caused by insufficient conversion, several improvements have been exploited, including optimizing bisulfite treatment conditions to increase deamination efficiency\textsuperscript{131,132}, using ACT random hexamers devoid of Gs to avoid copying inefficiently deaminated RNA templates\textsuperscript{133}, and developing robust computational pipelines to accurately identify m^7C sites\textsuperscript{132,133}. Ψ can be labeled by CMC at the Watson-Crick face, and the CMC-Ψ adducts stall reverse transcription, thus inducing truncations in cDNA synthesis. Combining this chemical reaction with next-generation sequencing, researchers developed Ψ-Seq, Pseudo-seq and PSI-seq, achieving base-resolution pseudouridylation detection in yeast and mammalian transcriptomes\textsuperscript{134–136}. To improve the robustness of Ψ detection, the chemical reaction is adapted by using a synthesized CMC derivative, azido-CMC (N\textsubscript{3}-CMC), instead of CMC. The presence of an azido group enables conjugation with biotin through a click reaction. Hence, the Ψ-containing RNA can be pre-enriched before sequencing, and this method is named CeU-seq\textsuperscript{137}. The pre-enrichment step enables CeU-seq to identify thousands of Ψ sites in the mammalian transcriptome (Fig. 4a). In addition to CMC labeling, a recent work showed that Ψ can form a stable monobisulfite adduct upon bisulfite treatment and further leave a deletion signature at the exact modified sites, thereby providing an orthogonal Ψ detection strategy (Fig. 4a)\textsuperscript{131}. As described above, m^7G residues can be converted to abasic sites upon NaBH\textsubscript{4} treatment and further recorded as misincorporations through reverse transcription and sequencing. Accordingly, the m^7G Mutational Profiling sequencing (m^7G-MaP-seq) can map internal m^7G modifications at nucleotide resolution in tRNA and rRNA\textsuperscript{138}. To achieve m^7G detection in mRNA, m^7G-seq conjugates a biotin molecule to the generated abasic sites, thus enabling pre-enrichment of m^7G-containing RNA. Moreover, the biotinylated sites induce misincorporations during cDNA synthesis; therefore, transcriptome-wide base-resolution m^7G mapping can be achieved (Fig. 4a)\textsuperscript{134}. In addition, D can also be reduced by NaBH\textsubscript{4} treatment, and the reduction product can be further labeled by Rho (rhodamine). Furthermore, the Rho-adducts block reverse transcriptase elongation and thus can be identified by analyzing induced RT-stops in sequencing data (Fig. 4a)\textsuperscript{139}. ac^C can react with NaCNBH\textsubscript{3} under acidic conditions, and the formed reduced nucleobase, N^6-acetylthreohydroxycytidine, causes misincorporation during cDNA
Taking advantage of this reaction, ac4C-seq can map ac4C at single-nucleotide resolution (Fig. 4a)\(^139\). In addition to altering the structure of nucleotides by chemical reactions, the chemical group can also be introduced through metabolic labeling. For instance, when feeding cells with an S-adenosyl methionine (SAM) analog, Se-allyl-L-selenohomocysteine, the cellular RNAs could be modified with N6-allyladenosine (a6A) at supposed m6A-generating sites. Furthermore, a6A-containing RNAs can be enriched by a specific antibody, and a6A sites are converted to N1,N6-cyclized adenosine (cyc-A) through the iodination-induced cyclization reaction. As cyc-A induces misincorporations in cDNA synthesis, m6A can be mapped at base resolution by detecting mutation signals in sequencing data (Fig. 3b)\(^140\). With the exception of metabolic labeling, the allyl group can also be transferred to m6A by the Dim1/KsgA family of dimethyltransferases, which can specifically convert m6A into allyl-modified m6A (N6-allyl, N6-methyladenosine, a6m6A). Since cyclized a6m6A can induce misincorporation in RT, the technology, named m6A-SAC-seq, achieves quantitative, transcriptome-wide mapping of m6A at single nucleotide resolution\(^141\).

Compared with unmodified nucleotides, modified nucleotides exhibit distinct resistance under chemical hydrolysis treatment. Based on this principle, several sequencing methods have been developed and have achieved transcriptome-wide profiling for Nm, m1G, m3C and Ψ. Nm is resistant to alkaline hydrolysis and thus can be mapped at single-base resolution by analyzing read-ends information in sequencing data. Since RNA fragmentation is random and irregular, this strategy requires rather high read coverage and is limited to highly abundant RNAs\(^142–144\). To overcome this limitation, ribose oxidation sequencing (RibOxi-seq) and Nm-seq have been developed. In these two methods, RNA is treated with iterative oxidation–elimination–dephosphorylation (OED) cycles to remove unmodified nucleotides, and the non-methylated ends cannot be ligated to linkers for sequencing library construction\(^145,146\). The selective cleavage and ligation allow detection of low-stoichiometry 2′-O-methylation sites using this approach. Similarly, m7G and m3C are resistant to NaBH4-aniline treatment, and the generated 5′-phosphate end during aniline cleavage could be exploited for selective ligation to enrich modified fragments\(^147,148\). This positive selection strategy facilitates transcriptome-wide detection of m7G and m3C. In addition, upon hydrazine-aniline treatment, RNA can be specifically cleaved at m3C-modified sites and resistant to cleavage at Ψ-modified sites. Based on this approach, hydrazine-aniline cleavage sequencing (HAC-seq) and HydraPsiSeq have been developed to detect m3C and Ψ, respectively (Fig. 4a)\(^149,150\).

**Antibody-based sequencing technologies**

Antibody-based strategies have been exploited for transcriptome-wide mapping of several RNA modifications, including m6A/ m6Am, m5A, hm5C, ac4C and m7G (Fig. 5)\(^39,53–55,148,151–154\). In this strategy, isolated RNA is first fragmented to 100–200 nt, and

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**Fig. 3** Schemes of direct sequencing technologies. a Detection of A-to-I editing sites by detecting A-to-G mismatches between genomic DNA and RNA sequencing (RNA-seq) data from the same individuals. b Detection of modified nucleotides by utilizing demethylase treatment, altered RT conditions, engineered reverse transcriptase, or modified dNTPs to generate distinct RT signatures at modified sites between treated and untreated samples. Modifications that can be detected by the corresponding approaches are listed below.
certain modification-containing RNA fragments are enriched by specific antibody immunoprecipitation. The enriched RNAs are subjected to high-throughput sequencing, and the modifications of interest can be identified by bioinformatic analysis. Robust enrichment allows antibody-based strategies to be very sensitive in detecting low-abundance modifications in mRNA and other rare RNA species.

However, limited by the fragmentation size, the resolution of the antibody-based strategy is approximately 100–200 bp. To improve the detection resolution, several improvements have been made. For instance, introducing UV-induced RNA-antibody crosslinking can lead to truncation or misincorporation at protein–RNA crosslinking sites during reverse transcription, thereby allowing transcriptome-wide single-base resolution.

**Fig. 4** Schemes of chemical-assisted sequencing technologies. **a** Schematic diagrams and sequencing features of different technologies. Corresponding technologies are listed below. **b** Flowchart of m6A-label-seq.
detection for m\textsuperscript{6}A and m\textsuperscript{7}G, respectively (Fig. 5a)\textsuperscript{155–157}. In addition, coupling antibody immunoprecipitation with enzyme/chemical treatment can not only increase detection resolution but also reduce false positives. For example, taking advantage of the fact that the m\textsuperscript{1}A-induced mutational RT signatures can be erased by AlkB demethylase treatment or Dimroth rearrangement, m\textsuperscript{1}A-MAP, m\textsuperscript{1}A-seq and m\textsuperscript{1}A-IP-Seq have achieved base-resolution m\textsuperscript{1}A methylome detection (Fig. 5a)\textsuperscript{42,122,158}. In addition, utilizing selective in vitro demethylation for m\textsuperscript{6}Am, m\textsuperscript{6}Am-seq has the capability of discriminating m\textsuperscript{6}Am from m\textsuperscript{6}A and can identify m\textsuperscript{6}Am at base resolution (Fig. 5b)\textsuperscript{159}.

**ENZYME/PROTEIN-ASSISTED SEQUENCING TECHNOLOGIES**

In addition to antibody immunoprecipitation, some enzymes or RNA modification-related proteins can also be utilized for affinity capture or editing modification-containing transcripts, thereby enabling transcriptomic RNA modification detection (Fig. 6). For instance, by feeding a 5-aza-C analog or overexpressing mutated Nsun2, a covalent bond can be formed between m\textsuperscript{5}C methyltransferase and its target sites. Based on this approach, Aza-IP (5-azacytidine-mediated RNA immunoprecipitation) and miCLIP (methylation iCLIP) can enrich target sites by immunoprecipitation, thereby enabling identification of the direct targets of m\textsuperscript{5}C methyltransferases (Fig. 6a)\textsuperscript{160,161}. In addition to methyltransferase, reader proteins can also be used to target modified nucleotides. For example, in DART-seq (deamination adjacent to RNA modification targets), the cytidine deaminase APOBEC1 is fused to the m\textsuperscript{6}A-binding YTH domain and thus leads to C-to-U deamination at sites adjacent to m\textsuperscript{6}A residues. Furthermore, m\textsuperscript{6}A residues can be identified by analyzing C-to-T mismatches in sequencing data (Fig. 6b)\textsuperscript{162}. Recently, the authors further integrated DART-seq with a single-cell RNA-sequencing platform and thus developed scDART-seq, achieving profiling of the m\textsuperscript{6}A methylome in single cells\textsuperscript{163}. In addition, some RNA exonuclease also possesses binding affinity for certain RNA modifications under certain conditions. For instance, in the presence of Ca\textsuperscript{2+}, E. coli Endonuclease V (eEndoV) promotes binding of inosine in RNA instead of cleavage. Taking advantage of this approach, Endonuclease V immunoprecipitation enrichment sequencing (EndoVIPER-seq) can enrich A-to-I edited transcripts from cellular RNA\textsuperscript{164}.

Similar to chemical-induced hydrolysis, some endonucleases also show distinct cleavage efficiency between modified and
unmodified transcripts, which can be utilized to enrich modification-containing transcripts and identify modified nucleotides. For instance, the *E. coli* RNA endoribonuclease MazF can specifically cleave the unmethylated 5′-ACA-3′ motif but not the 5′-m6ACA-3′ motif165. Taking advantage of this specificity, RNA-endoribonuclease–facilitated sequencing (m6A–REF-seq)/MAZTER-seq allows quantitative profiling of m6A at single-nucleotide resolution (Fig. 6c)166,167. However, a major limitation of this approach is that it can only detect m6A in the ACA context, which is only a small portion (16-25%) of m6A-modified sites.

**NANOPORE DIRECT RNA SEQUENCING-BASED DETECTION TECHNOLOGY**

Next-generation sequencing-based detection technologies have been widely applied for transcriptome-wide RNA modification detection. However, limited by the sequencing length (from 50 to 300 bp) and distinct detection principles for different modifications, next-generation sequencing-based detection technologies cannot map diverse RNA modifications simultaneously. The development of the Oxford Nanopore Technologies (ONT) sequencing platform shows promise in overcoming these challenges. In contrast to next-generation RNA sequencing, nanopore sequencing can sequence RNA directly without the requirement of additional reverse transcription and PCR amplification, thus decreasing the biases caused by these steps168–171. Mechanistically, single-stranded RNA is driven through the nanopore by the motor protein and thus causes ionic current changes for a set of k nucleotides residing within the pore (kmer; typically, k is 5), which enables decoding of the nucleotide sequence by computational analysis (Fig. 7). In addition to sequence information, chemical modifications and secondary structure, which also influence RNA translocation in nanopores, can be determined directly by computational algorithms172. In addition, the reads generated by nanopore sequencing are long enough to capture the full length of a transcript, thereby enabling accurate identification of highly repetitive regions, spliced products and polyadenylation tail length.

To identify RNA modification from the nanopore RNA direction, robust computational algorithm analysis is vital. There are two major analysis strategies, including alterations of raw signal intensity analysis (signal intensity, dwell time and trace) and base-called “error” feature analysis (base quality, mismatch frequency, and deletion frequency)170. Furthermore, modifications can be identified by the algorithms previously trained with modified and unmodified kmer contexts or comparison with the nonmodified control samples. These computational algorithms have allowed the identification of several modifications, including m6A, m3G, m3C, hm5C, Ψ and Nm, using nanopore direct RNA sequencing169,172–180. In addition, owing to the long sequencing length, the sequence information and modification landscape of SARS-CoV-2 can be determined simultaneously by nanopore direct RNA sequencing181,182. However, there are still many challenges and limitations for nanopore RNA sequencing-based detection technology. First, the sequencing error rate (~1–4%)168 is still much higher than that of next-generation sequencing (~0.1–1%). Since some algorithms exploit systematic base-calling errors to identify RNA modifications, the high sequencing error rate increases the complexity for base calling and modification identification. Second, the throughput of nanopore RNA sequencing is relatively low (1–3 Gb per flow cell), and the costs of direct RNA sequencing are high. Recently, several studies have shown that accurate modification detection requires high sequencing coverage (at least 30X)173,180. Hence, the sequencing depth and costs limit broader application of this strategy. Third, nanopore
The development of RNA modification detection strategies with single-cell RNA sequencing platforms shows promise. Collectively, recent progress in detection technologies promotes functional studies of RNA modifications, and we anticipate that further advancement will lead to a more comprehensive understanding of the epitranscriptome.

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