



Research review paper

Advances in the profiling of N⁶-methyladenosine (m⁶A) modifications

Hong-xiang Zheng ^a, Xian-sheng Zhang ^{b,*}, Na Sui ^{a,*}^a Shandong Provincial Key Laboratory of Plant Stress, College of life Sciences, Shandong Normal University, Jinan, Shandong 250014, China^b State Key Laboratory of Crop Biology, College of Life Sciences, Shandong Agricultural University, Taian, Shandong 271018, China

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ABSTRACT

Over 160 RNA modifications have been identified, including N⁷-methylguanine (m⁷G), N⁶-methyladenosine (m⁶A), and 5-methylcytosine (m⁵C). These modifications play key roles in regulating the fate of RNA. In eukaryotes, m⁶A is the most abundant mRNA modification, accounting for over 80% of all RNA methylation modifications. Highly dynamic m⁶A modification may exert important effects on organismal reproduction and development. Significant advances in understanding the mechanism of m⁶A modification have been made using immunoprecipitation, chemical labeling, and site-directed mutagenesis, combined with next-generation sequencing. Single-molecule real-time and nanopore direct RNA sequencing (DRS) approaches provide additional ways to study RNA modifications at the cellular level. In this review, we explore the technical history of identifying m⁶A RNA modifications, emphasizing technological advances in detecting m⁶A modification. In particular, we discuss the challenge of generating accurate dynamic single-base resolution m⁶A maps and also strategies for improving detection specificity. Finally, we outline a roadmap for future research in this area, focusing on the application of RNA epigenetic modification, represented by m⁶A modification.

1. Introduction

Over 160 RNA modifications have been identified, mostly in transfer RNA (tRNA) and ribosomal RNA (rRNA). Several modifications in mRNA have also been found, such as N⁷-methylguanosine (m⁷G) (Kiriaikidou et al., 2007; Malbec et al., 2019), N¹-methyladenosine (m¹A) (Dominissini et al., 2016; Li et al., 2016), N⁶-methyladenosine (m⁶A) (Dominissini et al., 2012; Luo et al., 2014; Meyer et al., 2012), 2'-O-dimethyladenosine (m⁶Am) (Sun et al., 2019), 5-methylcytosine (m⁵C) (Squires et al., 2012), 5-hydroxymethylcytosine (hm⁵C) (Delatte et al., 2016), inosine (I) (Levanon et al., 2004), 2'-O-methylated nucleosides (N_m) (Dai et al., 2017), uridylation (Chang et al., 2014), N⁴-acetylcytidine (ac4C) (Arango et al., 2018), and pseudouridine (Ψ) (Carlile et al., 2014). Increasing evidence suggests highly dynamic RNA modification has important roles in regulating the fate of RNA. Of these, m⁶A is the commonest functionally-important RNA modification. The m⁶A modification widely affects RNA metabolism, influencing RNA processing (Haussmann et al., 2016; Pendleton et al., 2017), RNA export (Edens et al., 2019; Roundtree et al., 2017), 3' processing of untranslated regions (UTR) (Bartosovic et al., 2017; Wei et al., 2018a), RNA stability (Huang et al., 2018b; Wang et al., 2014b), miRNA processing (Alarcón et al., 2015a; Alarcón et al., 2015b) and RNA translation (Zhang et al.,

2020; Zhou et al., 2015). Therefore, the accurate identification of m⁶A modifications within the transcriptome is crucial.

Two independent groups published methods of methyl-RNA immunoprecipitation, one combined with RNA sequencing, MeRIP-seq, (Meyer et al., 2012) and m⁶A-seq, (Dominissini et al., 2012). Both methods effectively identify m⁶A modifications within the transcriptome. MeRIP-seq and m⁶A-seq use antibodies to enrich m⁶A-modified RNA fragments taken from either total RNA or mRNA, followed by high-throughput sequencing. More recently, the so-called m⁶A-mapping method has described; it was based on epitranscriptome-mediated regulation of mRNA within cells, a concept now widely accepted. A plethora of m⁶A-mapping studies has revealed those transcripts which are regulated by m⁶A modification and also how m⁶A modification regulates gene expression within the cell.

Next-generation sequencing (NGS) provides a powerful tool able to identify low-abundance RNA modifications and explore their biological functions. Yet obtaining a detailed model relating m⁶A modification to gene expression remains a formidable challenge. Most studies of m⁶A-mapping, report averaged m⁶A modifications seen in a tissue or organ, without accurate cellular or subcellular detail, and with a relatively low resolution of approximately 100–200 nt (Dominissini et al., 2012; Meyer et al., 2012). m⁶A modifications seen in most studies are not

* Corresponding authors.

E-mail addresses: zhangxs@sdau.edu.cn (X.-s. Zhang), suina@sdnu.edu.cn (N. Sui).

stoichiometric, with the relationship between changes in stoichiometry and gene expression remaining refractory to investigation. m⁶A detection methods are now relatively mature, since m⁶A is the most widely studied RNA modification. Recent advances have seen NGS used to detect m⁶A modifications in combination with immunoprecipitation methods (Chen et al., 2015; Dominissini et al., 2012; Ke et al., 2015; Linder et al., 2015; Meyer et al., 2012; Molinie et al., 2016) and metabolic labelling methods (Hartstock et al., 2018; Shu et al., 2020). Enzyme-mediated methods have also been exploited widely. Such approaches can be separated into RT-mediated (Aschenbrenner et al., 2018; Harcourt et al., 2013; Hong et al., 2018), elongation-ligation-mediated (Liu et al., 2018; Xiao et al., 2018), and endonuclease-mediated (Garcia-Campos et al., 2019; Imanishi et al., 2017; Sednev et al., 2018; Zhang et al., 2019). Single-molecule real-time (SMRT) sequencing (Chen et al., 2019b; Vilfan et al., 2013) and single-molecule nanopore (Garalde et al., 2018) sequencing provide additional new ways to detect RNA modifications.

In this review, we focus on the latest advances in m⁶A-mapping. m⁶A-mapping methods are able to detect overall m⁶A modification levels, m⁶A sites, and the m⁶A transcription group range. We classify such methods here, based on their underlying m⁶A detection methodology, and evaluate their relative advantages and disadvantages. We also discuss the inherent challenges of accurately assessing dynamic m⁶A mappings at single-base resolution and current strategies for improving detection specificity. Finally, we explore different directions within future research and the potential applications of RNA epigenetic

m⁶A modifications. This review should prove useful as comprehensive primer for both experienced researchers and newcomers to the fascinating world of RNA modification, as well as acting as a powerful inspiration for those studying other forms of RNA modifications.

2. m⁶A antibody-based detection methods

Eukaryotic m⁶A mRNA modifications were first identified during the 1970s (Desrosiers et al., 1974; Schibler et al., 1977; Wei et al., 1975). Biochemical studies have shown m⁶A to be the commonest internal modification of mRNA, but a dearth of reliable analytical methods hampered proper investigation of its function and distribution (Dominissini et al., 2012; Luo et al., 2014; Meyer et al., 2012). In 2012, two groups developed independent antibody-based m⁶A detection methods: m⁶A-seq and MeRIP-Seq. Both methods, for the first time, mapped m⁶A modifications on mRNAs within the transcriptome (Fig. 1) (Dominissini et al., 2012; Meyer et al., 2012). m⁶A-seq remains the most commonly used approach to identifying m⁶A mRNA modifications. Researchers have improved MeRIP-Seq/m⁶A-seq based methods, enhancing the resolution of m⁶A identification and its stoichiometry (Chen et al., 2015; Ke et al., 2015; Linder et al., 2015; Schwartz et al., 2013).

Most RNA modifications are at low abundance. It is difficult to detect modifications on mRNA directly, due to single-stranded RNA's instability and structural complexity. During the reverse transcription (RT) of single-stranded mRNA into double-stranded cDNA, m⁶A and A (Adenine) cannot be distinguished accurately, resulting in loss of

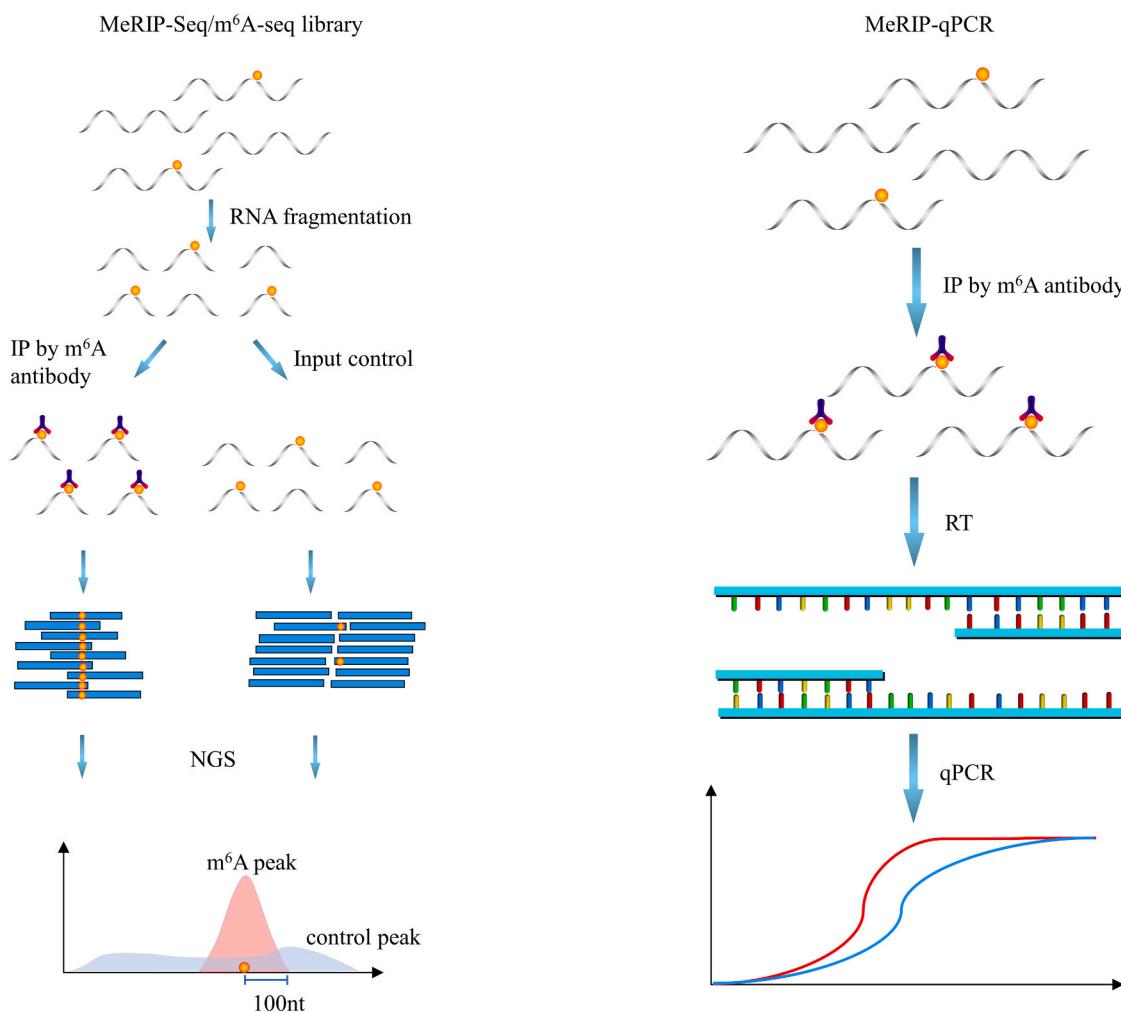


Fig. 1. Antibody-based m⁶A detection method.

a. Schematic diagram of the MeRIP-Seq/m⁶A-seq protocol; b. Schematic diagram of MeRIP-qPCR.

information concerning m⁶A RNA modification. The principal challenge in identifying m⁶A modifications is to distinguish them from unmodified mRNAs. Thus, enriching for m⁶A-modified RNA fragments and differentiating them from unmodified RNA is a key to all antibody-mediated methods.

MeRIP-Seq/m⁶A-seq requires two libraries: a MeRIP-Seq/m⁶A-seq library (IP, immunoprecipitation) and a RNA-seq library (input). The MeRIP-Seq/m⁶A-seq library incubates m⁶A-containing RNA with the antibody and then with beads. RNA-seq uses a conventional directly-constructed transcriptome sequencing library (Dominissini et al., 2013; Dominissini et al., 2012; Meyer et al., 2012). High-throughput sequencing (HTS) is used for both. The HTS read frequency increases as it nears the m⁶A site, creating a “peak” of approximately 200 nt at the bottom width and approximately 100 nt in the middle, which effectively labels the methylated transcript and its location (Fig. 1). Initial use of immunoprecipitation with NGS identified over 7000 mRNA m⁶A modification sites in human genes and more than 12,000 in mouse genes (Dominissini et al., 2013; Dominissini et al., 2012; Meyer et al., 2012).

MeRIP-Seq/m⁶A-seq is a straightforward and standardized process widely used for studying the role of m⁶A modification in regulating growth and development (Li et al., 2014; Wen et al., 2018; Yang et al., 2018; Zhou et al., 2019), disease (Lin et al., 2016; Vu et al., 2017), and stress resistance (Anderson et al., 2018). There are limits to the approach, such as the need for two libraries which can increase costs. Moreover, m⁶A sites can only be localized to an area of 100–200 nt, and so it is not possible to determine the precise number of m⁶A sites within the ‘peak’. To address this, library construction has been optimized. Resolution was increased by connecting adaptors to both ends of fragmented RNA and by reducing fragment size (Schwartz et al., 2013). To increase the acuity of the method, the original RNA-seq library was replaced by one without m⁶A writers (using mutants), as a negative control (Anderson et al., 2018; Schwartz et al., 2013).

Compared to m⁶A antibody-enriched RNA fragments and NGS, MeRIP-qPCR is more suitable for m⁶A identification at specific mRNA sites (Fig. 1) (Wang et al., 2014). MeRIP-qPCR uses premixed m⁶A antibody immunobeads to obtain full-length m⁶A modified mRNA from total RNA directly. The enriched RNA-antibody complex is first digested by protease to remove antibodies, and then the mRNA is subjected to RT-PCR. The method is both logically tractable and uses commercial kits to save time and labor. Combined with conventional RT-PCR, semi-chemical measurement of specific m⁶A sites is achievable. Moreover, for precious experimental samples, such as embryonic tissue, less RNA is needed for rapid detection of m⁶A modification. The MeRIP-Seq/m⁶A-seq method can identify m⁶A mRNA modifications, and then MeRIP-qPCR can verify the accuracy of HTS results while studying the function of m⁶A modifications on specific transcripts. The combination of MeRIP-Seq/m⁶A-seq and meRIP-qPCR is suitable for studying the majority of m⁶A mRNA modifications.

4-thiouridine (4SU) is a photoactive nucleoside analogue that can replace uridine in U-A pairing during transcription and improve the efficiency of cross-linking. In the PA-m⁶A-seq protocol (Chen et al., 2015), 4SU is added to the cell culture, allowing 4SU incorporation into newly synthesized mRNA in place of U. During the IP process, exposure to UV at 365 nm causes 4SU to cross-link with terminal aromatic amino acids of anti-m⁶A antibodies. Such a 4SU cross-link is read as C by RT-PCR or HTS, while the uncross-linked version as T. This approach reduces noise by selecting out PAR-CLIP clusters. Double filtering of m⁶A peaks and T-to-C transitions, which reduces peak clusters, improves the innate resolution of m⁶A modification detection. Using this method, the researchers mapped m⁶A modifications at a resolution of up to 23 nt (Chen et al., 2015; Liu et al., 2015; Xiao et al., 2016). During photo cross-linking, the 4SU transition in the PA-m⁶A-seq protocol can happen at protein binding sites of different transcripts, resulting in inconsistent quantitative measurements and inaccurate transition locations. In HTS, T to C mutation is partly random, thus adding a random component to m⁶A modification detection accuracy. In addition, constructing a PA-

m⁶A-seq library is complex, including the pretreatment of cells. The PA-m⁶A-seq approach is best suited to detecting m⁶A modifications in cultured cells *in vitro* and cannot easily be applied on a large scale.

Identification of specific m⁶A mRNA modifications at single-nucleotide resolution remains sub-optimal, hampering functional studies of m⁶A. In the miCLIP (Linder et al., 2015) and m⁶A-CLIP (Ke et al., 2015) protocols, PA-m⁶A-seq was optimized to remove cell pre-culture in 4SU (Fig. 2). Using this modified approach, purified RNA was fragmented directly, then incubated with m⁶A antibodies, and crosslinked using UV radiation at 254 nm. After affinity purification using protein A/G, covalently bound m⁶A antibody-RNA complexes were recovered using SDS-PAGE and nitrocellulose membrane transfer. Uncross-linked RNA is not transferred to the membrane, reducing background noise. RNA is released by proteinase K from the membrane and then subjected to RT and HTS. Peptide-fragments on RNA can cause nucleotide incorporation errors, which are displayed in HTS results as C-to-T over-transformation and shorter cDNA reads.

Previously, it was not possible to differentiate m⁶A and m⁶A_m modification as m⁶A and m⁶A_m have similar chemical properties and both bind 6-methyladenine antibodies. However, both m⁶A and m⁶A_m modifications involve many complex biological processes, and their precise location is crucial to understanding their function. By using different antibodies, miCLIP effectively distinguishes m⁶A from m⁶A_m. Recently, Jaffrey et al. optimized the miCLIP protocol, improving m⁶A or m⁶A_m mapping on the isotype transcript near the transcription start site (TSS) region (Hawley and Jaffrey, 2019).

Yet, miCLIP remains problematic: it is tedious, as the entire process takes an expert technician up to a week to complete. Additionally, many mRNA samples are needed as nitrocellulose membrane transfer and proteinase K elution are material intensive. Nonetheless, single-base-resolution of m⁶A modifications is achievable, making miCLIP an important tool for the study of m⁶A biological functions (Kan et al., 2017; Zhang et al., 2017).

m⁶A is an extensive and dynamic eukaryotic RNA modification regulating many aspects of RNA metabolism. Evidence suggest many genes give rise to two or more distinct transcripts encoding the same protein. m⁶A-modified transcripts may have a different fate to unmodified transcripts. Previous detection methods fail to detect how many gene transcripts are modified by m⁶A (“m⁶A level”) or determine accurately the ratio between m⁶A-modified and unmodified transcripts. Accurate quantifying the methylation state at a particular site is vital when exploring how m⁶A modification regulates RNA fate.

To facilitate accurate determination of the stoichiometry of m⁶A modification, Molinie et al. developed a method called m⁶A-level isoform-characterization sequencing (m⁶A-lAIC-seq) (Molinie et al., 2016). It differs from other antibody-mediated m⁶A tests in that it is undertaken directly with full-length transcripts for RIP as opposed to library construction without RNA fragmentation. In m⁶A-lAIC-seq strategy, the complete m⁶A IP positive transcripts and negative transcripts are directly subjected to HTS. Measurement of the m⁶A modification level is achieved by sequencing the full-length RNA of immunoprecipitation level and super-clear (Molinie et al., 2016). This method does not differentiate adjacent m⁶A sites, but avoids the many disadvantages of antibody-mediated methods, being an important step towards properly quantifying m⁶A levels (Molinie et al., 2016; Stranger et al., 2017).

3. Digestion-based detection methods

As the status of RNA alters as organisms grow and develop, so m⁶A modification levels will also change. The overall m⁶A modification level decreases during plant growth and development but can increase during period of stress (Li et al., 2018; Martínez-Pérez et al., 2017; Zhong et al., 2008). The m⁶A modification levels also differ between normal and cancer cells (Lin et al., 2016; Paris et al., 2019; Vu et al., 2017; Zhang et al., 2016). Methylated RNA detection and quantification can help to

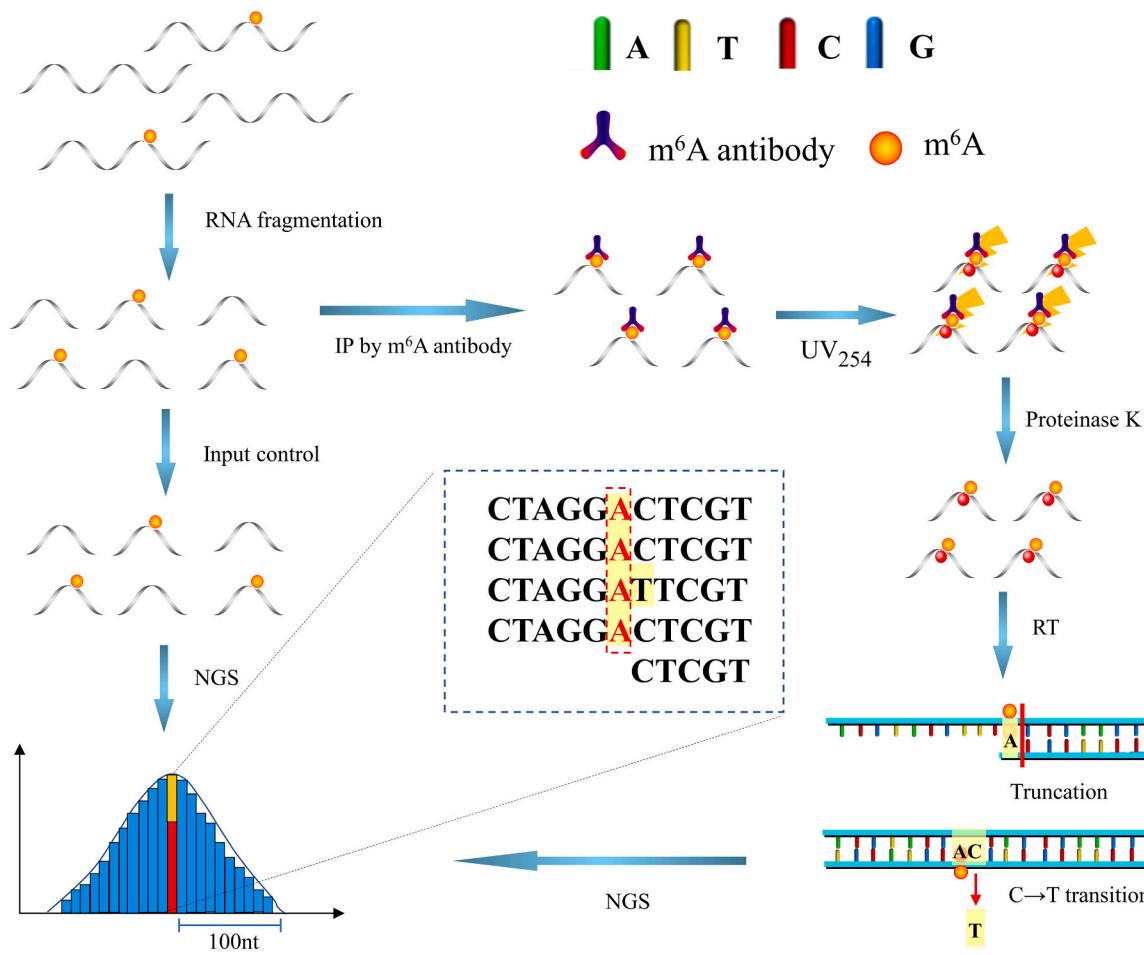


Fig. 2. Detection of m⁶A modifications within the transcriptome using the miCLIP/m⁶A-CLIP protocol.

deepen our understanding of certain cellular regulatory processes, including gene expression, immune responses, or epigenetic changes. Therefore, sensitive and accurate methods are needed to detect dynamic changes in overall m⁶A modification levels. Because modified (m⁶A) and unmodified nucleotides have different physical and chemical properties, use of liquid chromatograph mass spectrometer (LC-MS) based methods with single nucleotide digestion and ultraviolet detection, has become a standard quantitative method for assessing overall RNA modification levels (Thüring et al., 2017).

With its excellent selectivity and sensitivity, LC-MS has become widely used in qualitative and quantitative compound analysis (Fig. 3) (Tang et al., 2014). LC-MS is used primarily to study RNA modification at the nucleotide level. However, nucleic acids are typically long-chain biopolymers, thus it is first necessary to enzymatically degrade RNA to single nucleotides. RNase T1 and RNase A are the commonest RNA degradative enzymes, digesting RNA into small oligonucleotide fragments or single nucleotides. Single nucleotides can be compared with standard nucleotides to quantify the proportion of m⁶A present. LC-MS is currently the standard method for detecting overall m⁶A modification levels because it is simple to operate yet highly sensitive (Thüring et al., 2017). However, this method cannot determine the RNA position of detected m⁶A modifications. Nor can it distinguish m⁶A on mRNA from that on rRNA or snRNA contaminants, if tested directly after digestion with total RNA. Thus, most samples need to be enriched for mRNA, and purified. It is important to emphasize this method is able to detect multiple RNA modifications simultaneously using standards or a database of RNA modifications.

At single nucleotide resolution, modified mRNA and lncRNA is difficult to detect due to the low abundance of different modifications.

Traditional deep sequencing does not identify low-abundance mRNA/lncRNA modifications. Thin layer chromatography (TLC) has been used to analyze nucleotides, nucleosides, and bases for over 50 years (Zhong et al., 2008). TLC analysis of RNA modification is both qualitative, comparing the migration rate of sample and corresponding controls, and quantitative, measuring spot intensity. TLC separation can be either one- or two-dimensional (2D-TLC). 2D-TLC provides better separation and has been used widely to analyze RNA modifications (Bodi and Fray, 2017).

Due to TLC's low sensitivity, it cannot be used to detect low-abundance RNA modifications. Moreover, when combined with a radioisotope, its sensitivity is improved using autoradiography detection. Zhong et al. used ³²P-labeled 2D-TLC to identify m⁶A modified mRNA in *Arabidopsis*, showing the *Arabidopsis* m⁶A content was similar to that of animal cells (Bodi and Fray, 2017). Similarly, Liu et al. developed a method of site-specific cleavage and radiolabeling followed by ligation-assisted extraction and TLC (SCARLET), which detects m⁶A modified mRNA and lncRNA accurately and quantitatively (Liu et al., 2013). Compared to MeRIP-qPCR, SCARLET is more sensitive and suitable for detecting low-abundance m⁶A modifications at specific sites. However, it involves multiple enzymatic transformations and separations which are very labor intensive, require expensive reagents, and poses a radiological hazard. These demanding technical requirements have limited the wide application of SCARLET.

Antibody-mediated methods have been used widely to identify m⁶A modifications. However, due to variable antibody affinity and batch effects, it has poor reproducibility and limited resolution. Moreover, enhanced versions of this methodology need complicated database construction. As a consequence, a simple, antibody-independent method

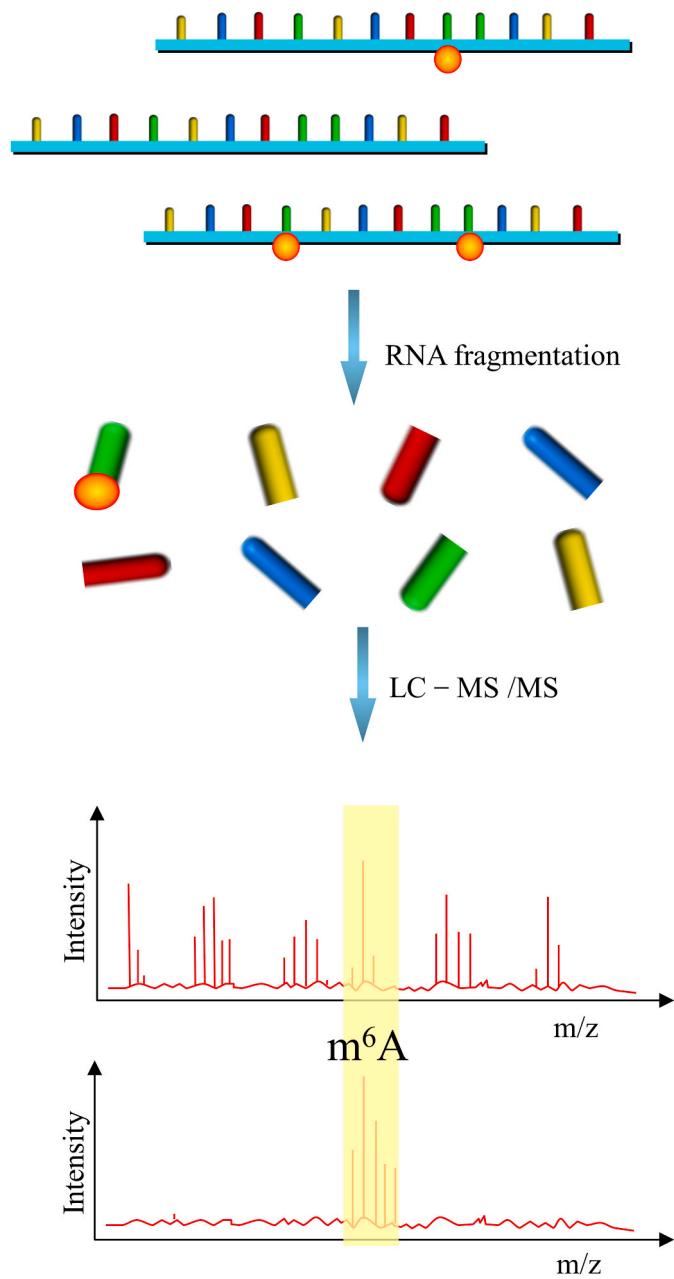


Fig. 3. Detection of m^6A modification at the overall level by LC-MS/MS protocol.

able to cross-validate known m^6A sites and identify the single-nucleotide location of m^6A modifications is needed. The selective digestion of nucleotide modification sites, characteristic of several endonucleases, combined with NGS, at single-base resolution allows detection of DNA N⁶-methyladenine modification (6 mA) across the genome (Luo et al., 2016).

Miki et al. identified a sequence-specific endoribonuclease called MazF in *E. coli*. MazF is sensitive to m^6A modification, cleaving RNA at the 5' end of ACA motifs, but not RNA with an m^6A modified (m^6CA) motif (Fig. 4) (Imanishi et al., 2017). Using m^6A restrictive mazF RNA cutting and NGS, two separate groups developed MAZTER-seq (Garcia-Campos et al., 2019) and m^6A -REF-seq (Zhang et al., 2019). Both are simple compared to antibody-mediated m^6A detection, as they both only involve MazF digestion of purified mRNA, followed directly by high-throughput sequencing. m^6A modifications are identified by analyzing if restriction sites are internal to the read or are at the end. RNase-based

differential cleavage may generate read discrepancies during HTS. Quantitative site-specific m^6A modification analysis calculates the difference between cut and uncut reads. Due to MazF's high sensitivity and specificity, m^6A and m^6Am can be distinguished. Use of m^6A demethylase FTO-treated mRNA *in vitro* as a negative control significantly reduces false positives (Garcia-Campos et al., 2019; Zhang et al., 2019).

However, this method has some obvious limitations. MazF only recognizes the ACA motif; this accounts for only 16–25% of the classic m^6A RRACH motif, so cannot identify all m^6A sites. During digestion, as well as ACA cleavage, some additional cleavage was observed for similar sequences, such as AGA or AAA. This "off-target activity" may result in identification of m^6A modification near the off-target site. Nevertheless, this method for detecting m^6A is not antibody-mediated. Due to library construction being simple and its high innate sensitivity and specificity, this method may be used for rare biological materials, such as pathological tissue or early embryos. Additionally, MazF, directed evolution, or other screening methods, can identify endonucleases recognizing the classic DRACH motif of A and m^6A . This efficient yet convenient approach should greatly expand the scope of m^6A research (Garcia-Campos et al., 2019; Zhang et al., 2019).

Sednev et al. recently described another endoribonuclease (Sednev et al., 2018), sensitive to m^6A modification close to the cleavage site and which can cut m^6A -modified RNA more rapidly than MazF. It has also been shown to cleave natural RNA sequences, such as lncRNA. Using this new m^6A -sensitive endoribonuclease, combined with NGS, it may be possible to identify m^6A modifications in MazF-seq, in addition to those operating on the ACA motif.

4. m^6A sensing reverse transcription (RT)-based detection methods

A major obstacle to detecting m^6A RNA modification using HTS methods is that conventional reverse transcriptases are insensitive to m^6A modification. This may result in base mismatches, which induce mismatch at the modified site or the early termination of the transcript. By combining existing methods with RT-PCR or HTS to analyze such mismatched bases or the early termination of cDNA, it may be possible to obtain full locations for all RNA modifications. Thus, m^6A modified sites or single nucleotides, with different pairing abilities, or screening using m^6A modified site sensitive RT, provides new ways to detect m^6A modified RNA.

Polymerases and reverse transcriptases have spatially-sensitive catalytic sites, allowing screening for reverse transcriptases or polymerases sensitive to m^6A modification able to detect modification sites. Harcourt et al. screened Tth DNA polymerase, which is sensitive to m^6A modification, from *Thermos thermophilus* (Harcourt et al., 2013). During transcription, Tth DNA polymerase preferentially promotes dTTP pairing with A rather than m^6A (Fig. 5b) (Harcourt et al., 2013). T-A incorporation is 4–18× more selective than incorporation of T- m^6A . So m^6A modifications in RNA can be located and quantified by analysis of the pause band. Comparison to data using a non-selective polymerase indicates the Tth DNA polymerase can locate high-abundance m^6A modification sites. However, the pairing of T-A or T- m^6A by Tth DNA polymerase is constrained by reaction conditions, such as reaction time, enzyme concentration, and RNA concentration, producing different results for different samples or different mRNAs. This method only detects m^6A modification at one RNA site at a time, and thus cannot be used for HTS. However, it does provide a relatively facile method which is quantitative in m^6A detection, even when mRNA abundance is low.

Certain RNA modifications block the RT process generating increased mismatches in nucleotide incorporation and/or RT termination at the modification site, which allows direct prediction of N¹-methyladenosine (m^1A) modified loci from NGS alone (Hauenschild et al., 2015). It is known that certain RT active DNA polymerases can distinguish m^6A from unmodified A (Harcourt et al., 2013). Thus, using retroviral modification or mutation, a DNA RT polymerase may be able

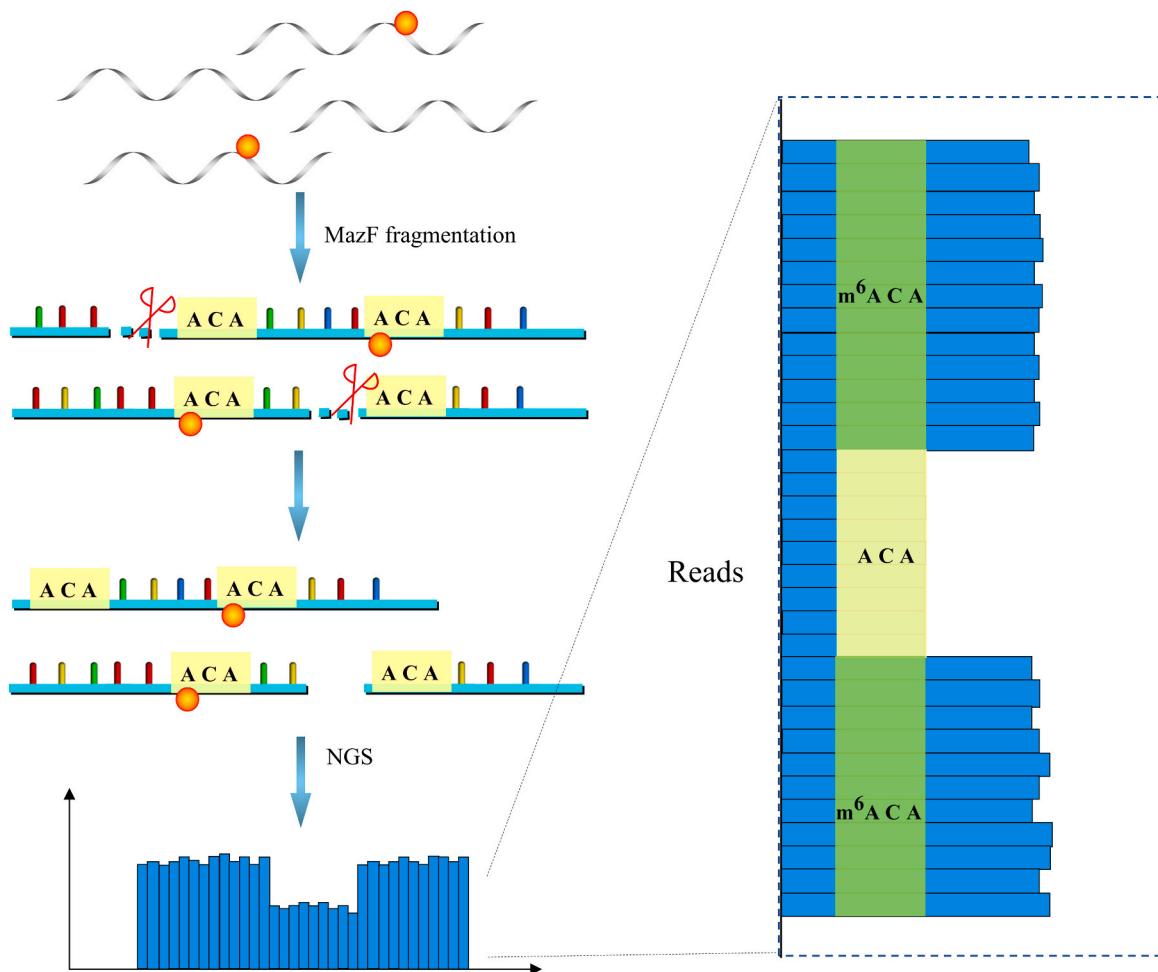


Fig. 4. Detection of m⁶A modifications within the transcriptome using MAZTER-seq and m⁶A-REF-seq protocols.

to distinguish m⁶A modification. Increasing the error rate of m⁶A and A during RT, combined with NGS, can be used to detect m⁶A modification. Joos et al. made point-mutants of the RT-active heat-resistant KlenTaq DNA polymerase variant (KlenTaq L459M S515R I638P M747K), obtaining polymerase mutants sensitive to m⁶A modification (Fig. 5b) (Aschenbrenner et al., 2018). A mutation with high specificity for m⁶A modified RT was screened. Using this, m⁶A sites were identified using an increased rate of mismatch errors in m⁶A. This strategy may assist development of methods capable of directly detecting m⁶A sites in cellular RNA using RT-binding NGS. However, this method may have a high false positive rate due to the unquantifiable blocking effect of m⁶A. Moreover, due to database construction limitations, this method cannot detect m⁶A modifications in the 5' UTR region. Nonetheless, it provides a way of detecting m⁶A modification using a simple experimental protocol and is independent of the need for antibody enrichment (Wang et al., 2014b).

As m⁶A is chemically stable, mutations or truncations cannot be introduced during RT. Substituting atoms in the original base with elements of the same group of the periodic table may be an effective way of altering the properties of the resulting modified base, interfering with its ability to undergo base-pairing. Hong et al. replaced the oxygen at the 4-position of deoxythymidine triphosphate with sulfur or selenium to obtain a T* with reduced base-pairing ability with m⁶A, while the T* to A pairing remained unaltered (Fig. 5a) (Hong et al., 2018). Thus 4SeT can successfully pair with A during RT. Yet, 4SeT cannot pair with m⁶A due to the disruption of hydrogen bonding and base stacking. Unfavorable m⁶A-4SeT base pairs cause RT to stall, stopping cDNA synthesis at the m⁶A site. By designing PCR primers on either side of this site, it is

possible to determine if the site has a m⁶A modification. Thus, by using m⁶A demethylase (FTO) and NGS, accurate m⁶A identification is possible at single nucleotide resolution.

However, this method also has a high false positive rate, particularly when analyzing m⁶A distribution in an unknown region, and has not been used for m⁶A modification identification. Nevertheless, the atom substitution strategy provides a way to identify epigenetic modifications, not typically sensitive to chemical transformations and is not reliant on antibody-mediated methods.

5. Ligation-based detection methods

Although m⁶A sequencing provides information concerning the whole transcriptome, studying m⁶A's biological function can require identifying specific m⁶A modifications on a particular transcript. Compared to DNA polymerase RT-based methods, DNA ligase has greater specificity, detecting specific transcripts by amplifying ligated m⁶A-modified DNA products.

Liu et al. screened a selective commercially-available m⁶A-sensitive ligase, T3 DNA ligase, able to distinguish m⁶A from A (Liu et al., 2018). T3 DNA ligase ligates an RNA template to a DNA probe. When there is an m⁶A modification near the detection site, the probe prevents linking, which decreases probe incorporation. When qPCR amplification is performed, ligation and final PCR products generated by m⁶A-modified transcripts will be greatly reduced. Similarly, Xiao et al. identified an enzyme, SplintR, sensitive to m⁶A-modification, and established a single-base elongation and ligation-based qPCR method (SELECT), able to identify m⁶A modifications at specific sites within a single transcript.

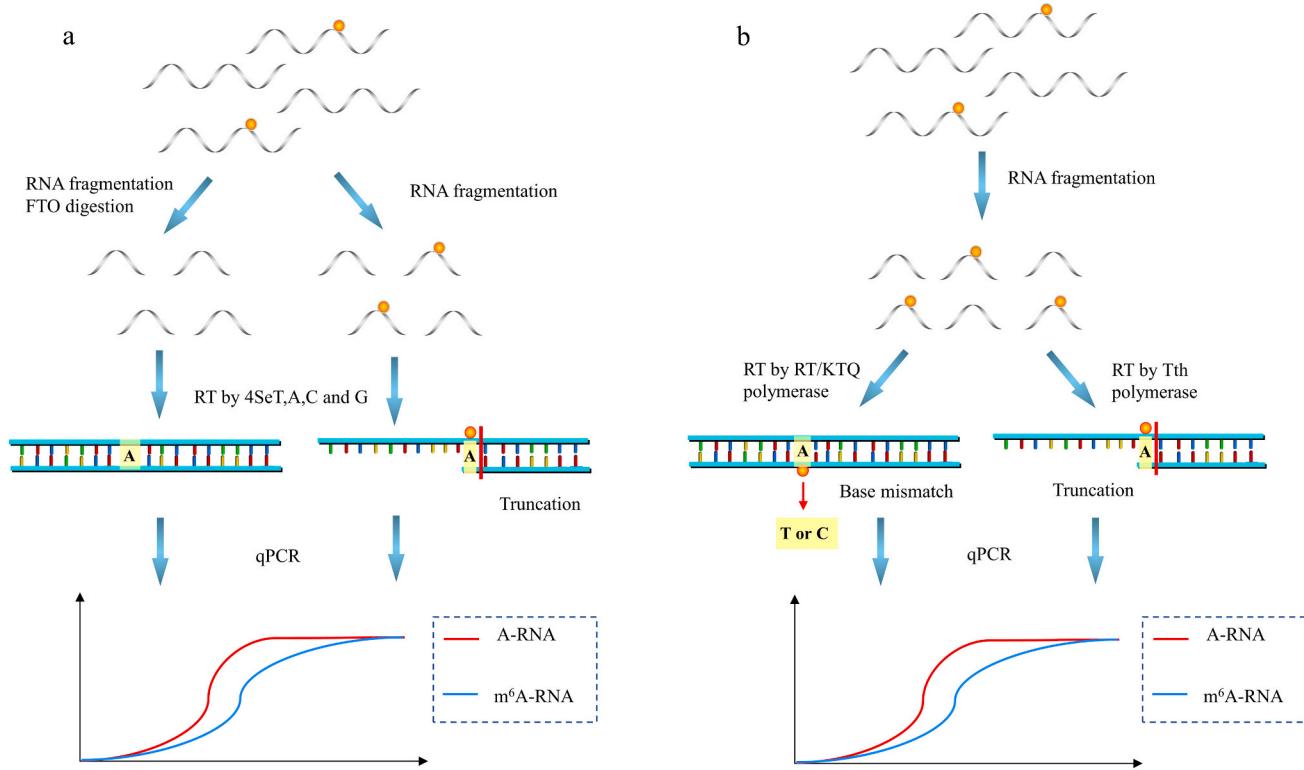


Fig. 5. a. Detection of m⁶A modification using 4SeTTP and FTO assisted strategies; b. Schematic diagram of RT-sensitive m⁶A detection method.

(Xiao et al., 2018). Compared to the T3/T4 DNA ligase-qPCR method, SELECT (Xiao et al., 2018) has additional reverse transcription steps which modify the sensitive reverse transcriptase Bst DNA polymerase with m⁶A (Wang et al., 2016). m⁶A-modified RNA templates are

selectively obstructed during Up Probe monobase extension, as mediated by Bst DNA polymerase. The Splint R enzyme then make a secondary selective connection to the m⁶A modified site. After two rounds of m⁶A selection, the final number of m⁶A connection products is greatly

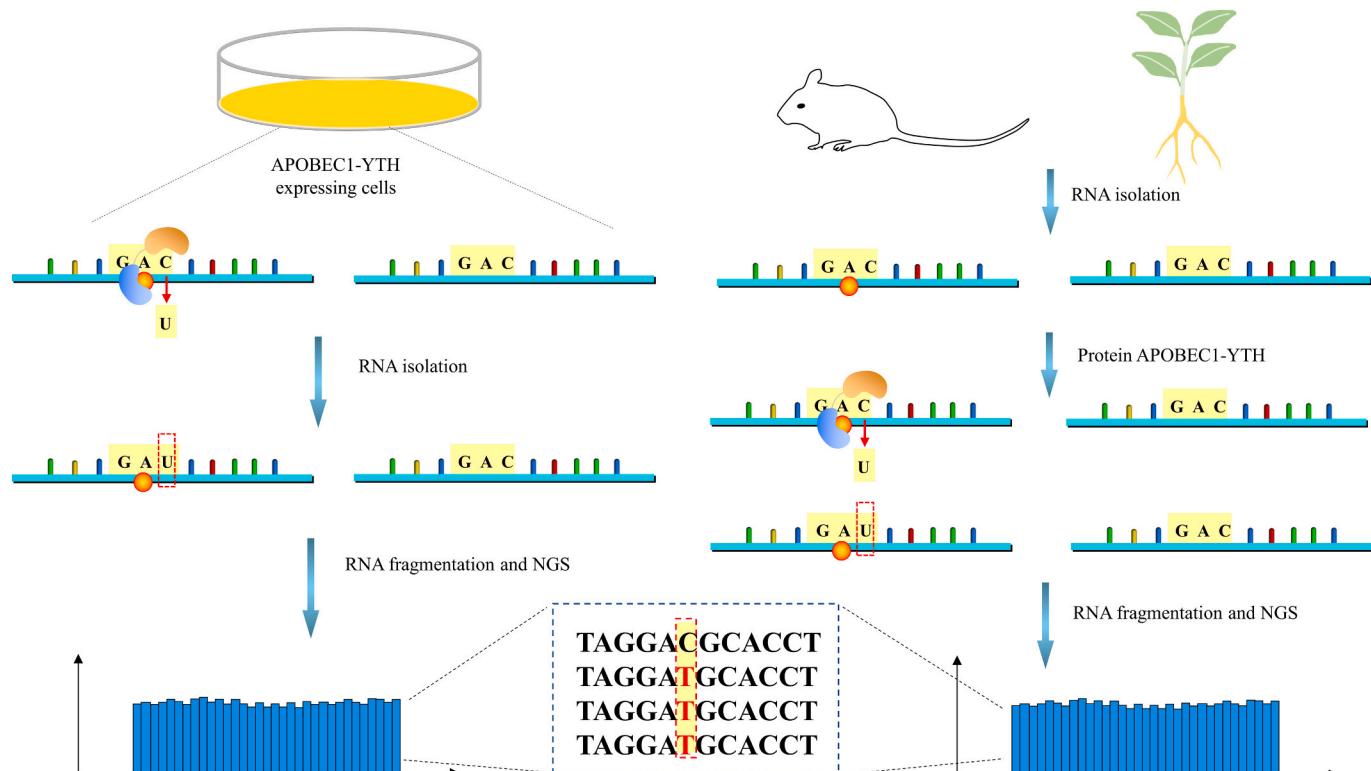


Fig. 6. DART-seq strategy detects transcriptome-wide m⁶A modifications.

reduced, when compared with those formed by unmodified RNA templates. m^6A -modified and unmodified target templates can then be easily quantified using qPCR.

Compared to m^6A -sensing RT-based methods, ligation-based detection has higher sensitivity and can be used to detect m^6A quantitatively in low-abundance cellular RNA samples. The SCARLET method needs radioactive reagents and has a complex operating procedure, while ligase-dependent PCR assays need only routine laboratory resources and are relatively fast; SELECT requires only 3 h for example (Xiao et al., 2018). Thus, as well as HTS, ligase-dependent PCR assays provide a fast, low-cost method for the quantitative detection of single-transcript m^6A modifications. Ligase-dependent PCR assays can be used effectively in both pure research and clinical diagnosis.

6. Gene editing-based detection methods

The APOBEC1 cytosine deaminase decarboxylates Cytosine into Uracil. The YTH protein family of so-called m^6A readers bind selectively to m^6A modification sites. When APOBEC1 and YTH are fused, APOBEC1 can locate m^6A sites helped the YTH domain, changing C to U after m^6A in the Rm 6A CH motif (Meyer, 2019). RNA can then be extracted, still bound to the APOBEC1–YTH fusion protein, and sequenced. The C-to-U site, occurring after A, can be detected, indicating an m^6A modification at this site (Fig. 6) (Meyer, 2019).

The deamination adjacent to RNA modification targets, or DART-seq, method has several advantages over previous approaches. It only requires 10 ng of RNA, about 1/50th of the required sample size for MeRIP-Seq. This reduced sample size allows it to be used to analyze precious samples or m^6A modifications in single-cells. If a change occurs in APOBEC1-YTH's subcellular localization, perhaps resulting from the addition of different localization signals, APOBEC1-YTH may still accurately detect m^6A modifications in specific regions or in cellular organelles (Meyer, 2019).

7. Metabolic labeling-based detection methods

Attempting to exploit the metabolic pathways responsible for m^6A synthesis, Shu et al. reported an m^6A -label-seq strategy which replaced the N 6 -methyl group with other modifications that were easier to identify and to purify, thereby detecting m^6A mRNA modification (Shu et al., 2020; Shu et al., 2017). S-adenosine methionine (SAM) is a co-factor of methyl transferase able to transfer its methyl group to the N 6 -position of a specific adenine on mRNA, and thus generate an m^6A modification via the action of m^6A methyl transferase (METTL3/METTL14). SAM is synthesized from methionine and adenosine triphosphate (ATP) catalyzed by methionine adenosine transferase (MAT). In the m^6A -label-seq strategy, allyl-methionine is converted by MAT to allyl-SAM or its selenium homolog allyl-SeAM. This allyl-substituted cofactor, when acted on by METTL3/METTL14, replaces the original mRNA m^6A site to generate N 6 -allyladenosine (a 6A). On iodine addition, a 6A induces formation of cyclized N 1 , N 6 -cyclized adenosine (cyc-A), resulting in base mismatch during reverse transcription. Through HTS and bioinformatics analysis of mutation sites, an m^6A map at single-base resolution can be obtained.

Similarly, Hartstock et al. developed a direct chemobiological detection method for m^6A modification sites using a synthetic analogue of S-adenosyl-L-methionine (AdoMet) (Hartstock et al., 2018). In this approach, METL3-METTL14 uses selenium-based AdoMet analog Se-AdoYn as a co-substrate to catalyze N 6 -propargylation of adenosine, generating N 6 -propargyl-adenosine (p 6A). However, p 6A demonstrated only weak RT termination, so Hartstock et al. increased termination by enlarging the N 6 -adenosine modification by reacting the propylated RNA with biotin azide in a copper-catalyzed azide-alkyne cycloaddition (CuAAC). By combining this with NGS, an m^6A modification map can be obtained. For bioconjugated p 6A -RNA, this approach can localize m^6A modifications to a 1-2 nt resolution. Compared to N 6 -allyl adenosine

iodization-based methods, the greatest advantage of this protocol is the high selectivity of the CuAAC reaction used to enrichen RNA, which might otherwise be m^6A modified, improving the detection efficiency.

The greatest advantage of metabolic labeling-based methods over the antibody-mediated methods is that the m^6A mRNA modification can be detected directly. Errors due to differences in distance between the antibody cross-linking points and m^6A sites is avoided, and multiple m^6A modifications at similar positions can be identified. A key stage in this protocol is the pre-culture of cells in mediums containing Se-allyl-l-selenomocysteine or Propargyl-L-selenohomocysteine, in which easy-to-detect a 6A or p 6A replaces m^6A . There are also certain unavoidable limitations, such as low labeling yield, meaning all potential m^6A modifications cannot be identified. Cells can generate a stress response, affecting the methylation process, resulting in changes to detected m^6A modifications. Moreover, test results are affected by the conditions and duration of pre-cultivation, which may produce so-called “batch effects”. Nonetheless, metabolic labeling has opened up a new way to detect m^6A modifications at single-base resolution.

8. Direct RNA-based detection methods

High-throughput technology enables rapid HTS, but involves sample amplification, which may produce errors or the loss of base modification information. Third generation sequencing, which does not require sample amplification, can be used for single-molecule sequencing and direct RNA modification location analysis. Such approaches include SMRT sequencing (Flusberg et al., 2010; Vilfan et al., 2013) and nanopore DRS (Xu and Seki, 2020).

SMRT differentiates distinct DNA modifications by monitoring DNA polymerase in real time to distinguish the different dynamic signals of normal and modified bases on the template (Flusberg et al., 2010). Korlach et al. improved SMRT, successfully locating m^6A -modified RNA sites using RT combined with SMRT (Vilfan et al., 2013). Using this approach, where DNA polymerase is replaced by HIV reverse transcriptase, RT signals can be monitored in real time. Modified bases are typically less able to carry fluorescent markers than unmodified bases, and so each modification has its own “kinetic signature”. The dynamic signal of RT encountering m^6A is significantly different to A in control RNA, and the pulse frequency at the m^6A position is significantly lower than unmodified A. This method is also suitable for location analysis of many other modifications (Vilfan et al., 2013). However, SMRT's location analysis is mainly confined to synthetic RNA, and analysis of real-world clinical samples needs considerable improvement.

As well as SMRT sequencing, nanopore DRS technology can also be used for single-molecule nucleoside detection (Cherf et al., 2012; Noakes et al., 2019; Xu and Seki, 2020). Nanopore DRS uses voltage to drive molecules through nanopores, where different nucleosides induce different currents, allowing normal and modified nucleosides to be distinguished using current changes. This approach does not require enzymatic synthesis and has the potential to analyze nucleic acid chains directly. Nanopore DRS has been widely used to detect DNA modification directly (Xu and Seki, 2020).

Ayub et al. were able to identify RNA bases in oligonucleotides using α -hemolysin (α HL). By monitoring current changes, the four biogenic RNA nucleobases and various modified bases, including I, m^6A , and m^5C , could be distinguished (Fig. 7) (Ayub and Bayley, 2012). Moreover, Oxford Nanopore Technology (ONT) has extended DNA nanopore sequencing to RNA, allowing directly RNA modifications on RNA sequencing (Garalde et al., 2018). Due to the single-molecule nature of nanopore DRS, this technology would be well-suited to study samples small in size, with the potential to accelerate the research of m^6A .

Direct detection of RNA modification using nanopore sequencing has many advantages over NGS. No PCR amplification is required during sequencing, so there is no PCR bias. Direct sequencing of full-length RNA allows the study of the relationship between m^6A modifications and spliced transcripts (Parker et al., 2020). It can also directly mark

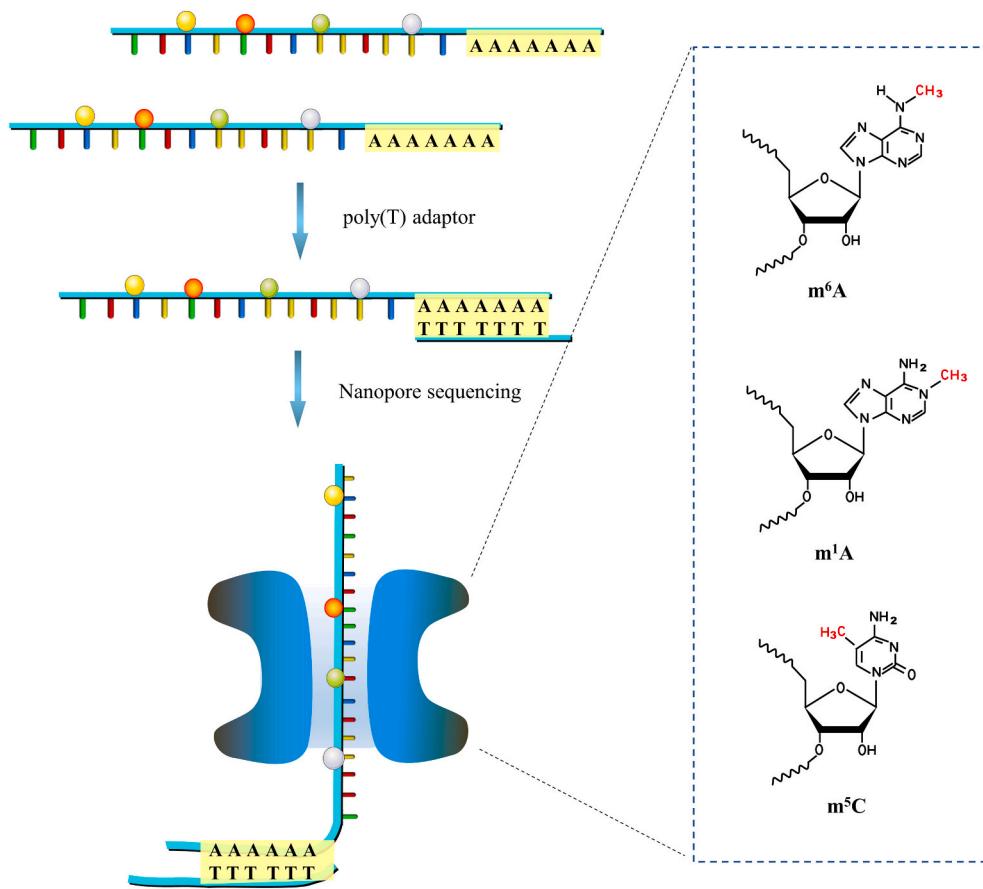


Fig. 7. Schematic of the direct detection of m^6A modification by nanopore sequencing.

multiple m^6A modification sites on a single RNA. This method also has the potential to detect multiple RNA modifications simultaneously in one experiment.

However, as RNA strands can fold into complex secondary

structures, such structures must be eliminated before nanopore sequencing. RNA is also unstable and is easily degraded, so the sequencing of long RNA strands may generate inaccurate results. Some RNA modifications cause only small current changes, making it difficult

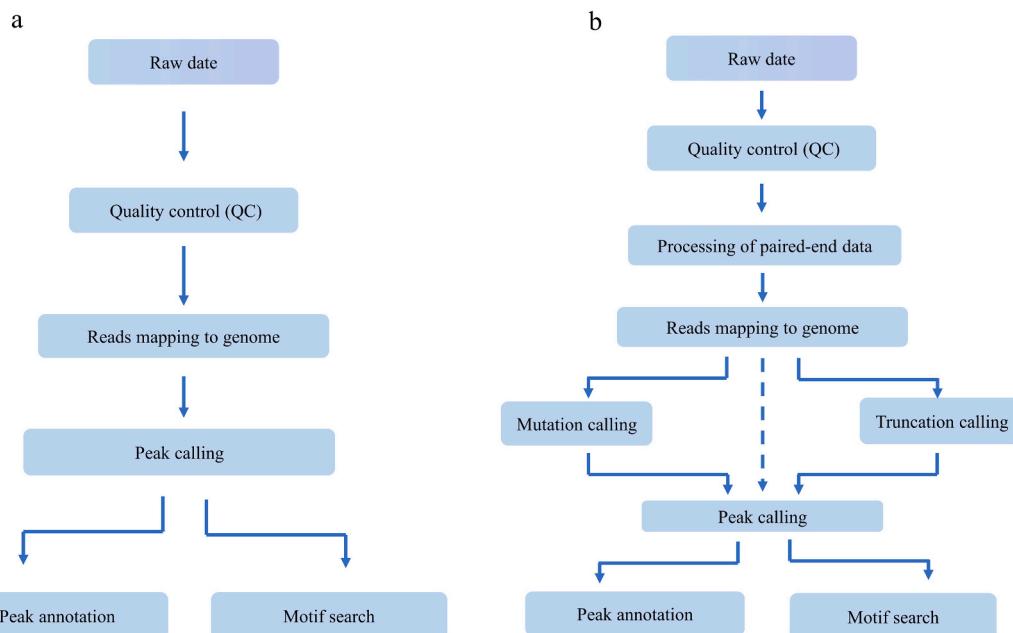


Fig. 8. Example workflow for analysis of m^6A data. a. Analysis of m^6A -seq data; b. Analysis of single-resolution m^6A detection methods data based on truncated or mutated by RT.

to distinguish accurately between certain modifications, and potentially leads to high false positive rates. To address the problems in nanopore DRS, researchers are actively exploring options for improvement. Liu et al. developed a computational method for EpiNano by exploiting the property that RNA modification causes an increase of “error” in the output of the base-calling algorithm (Liu et al., 2019). EpiNano used the “error” detected by the base as an identification m⁶A modification with a prediction of 90% accuracy. The increased usefulness of nanopore DRS may depend on more accurate base-calling, refined splitting of signal, and more information processing software development. Predictably, nanoporous sequencing technology will greatly facilitate the positioning of a variety of RNA modifications, including m⁶A, on mRNAs.

9. Brief overview of computational-based methods

Together with advances in RNA epigenetics, especially new ways of profiling the RNA methylome, computationally deciphering the transcriptome using so-called omics data, presents a major challenge to bioinformatics. Below, we review commonly used m⁶A-orientated computational approaches.

In the m⁶A-seq protocol (Fig. 8a), FASTP (Chen et al., 2018) and FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc>) is used for quality control (QC), removing reads that contain adaptor contamination, low quality bases, and undetermined bases. HISAT2 (Kim et al., 2015) was used to map reads to the reference genome. Mapped IP reads and input libraries are available for the R package exomePeak (Meng et al., 2014) which can identify m⁶A peaks in “bed” or “bigwig” format, which can be adapted for visualization using IGV software (Robinson et al., 2011; Thorvaldsdóttir et al., 2013). HOMER (Heinz et al., 2010) was used for *de novo* motif finding, followed by motif localization relative to the peak. Called peaks are annotated using the R package ChIPseeker (Yu et al., 2015). Next, StringTie (Perteet et al., 2015) was used to calculate expression levels for all mRNAs from input libraries.

Other improved methods based on m⁶A-seq (such as miCLIP/m⁶A-CLIP) and other single-resolution m⁶A detection methods are usually truncated or mutated at the m⁶A modification site during the RT process, so the computational approaches are generally similar to m⁶A-seq. During data analysis, these methods add the steps of “Mutation calling” by CIMS (Zhang and Darnell, 2011), “Truncation calling” by CITS (Weyn-Vanhentenryck et al., 2014), and perform “Peak calling” at the same time to filter the results to improve accuracy and resolution (Fig. 8b).

10. m⁶A site prediction software tool guide

Although m⁶A is functionally important, its catalytic mechanism remains poorly understood. Recently, progress in m⁶A-mapping has resulted in the rapid accumulation of m⁶A modification datasets. Nevertheless, the identification of RNA methylation sites and their functions using wet laboratory experimentation is still very expensive in terms of time, labor, and resource. By contrast, bioinformatics may be able to predict the probable function of single RNA methylation sites using just statistical evidence.

Many studies indicate m⁶A modification exhibits regularity: such as its preference for the “RRACH” motif, where R = A/G, A is the modified m⁶A site, and H = A/C/U. By integrating sequence and structural features around m⁶A sites using machine learning, reliable prediction of m⁶A modification can be achieved. This advance has led to more dependable tools for biological macromolecule identification, greatly reducing experimental cost.

To date, many RNA methylation prediction methods and servers have been developed, including SRAMP (Zhou et al., 2016), M⁶APred-EL (Wei et al., 2018b), WHISTLE (Chen et al., 2019a), etc. These m⁶A site predictors typically use a transcript sequence as input, reporting back possible m⁶A sites, making such server implementations highly

convenient. It should be emphasized that m⁶A modification has clear species and tissue specificity. Due to different test and training sets, the accuracy of different predictors varies, particularly across species. Most predictors are currently developed using m⁶A modification data from humans and mice. We have undertaken a detailed comparison of commonly used predictors (Table 1) to help assess the relative performance of different methods and implementation.

The rapid accumulation of public m⁶A modification datasets means effective query methods are essential to the efficient management and annotation of newly-sequenced m⁶A data. By integrating and annotating existing public datasets, batch effects may be minimized. Moreover, public databases can be linked to data from Genome-Wide Association Studies (GWAS), ClinVar, and RNA-binding protein (RBP) interactions (Deng et al., 2020; Luo et al., 2020; Wu et al., 2019; Xuan et al., 2017), amongst others, greatly assisting in the discovery of potential functions of RNA modifications and the exploration of relationships between m⁶A and RBP and disease (Table 2). Appropriately used, public data sets will aid researchers, especially newcomers or interdisciplinary scientists, greatly reducing the costs of preliminary investigation, and also aid selection of appropriate experimental programs.

11. Design and analysis of m⁶A experiments

Choosing a suitable method to detect m6A sites is typically only the first step in research on the function of m⁶A modification. Here, we compare comprehensively available detection methods for m⁶A modification (Table 3). Account must be taken of many factors when choosing a detection method, including *inter alia* cost, difficulty of use, duration of operation, and sample requirements. It should be emphasized that simply choosing the latest method is not always best, instead choose the most suitable method as dictated by the experimental. Below, we discuss how to use predictors and public databases to design optimal experimental protocols (Fig. 9).

Previously, researchers have investigated the function of m⁶A modification by knocking down or overexpressing m⁶A modification enzymes before performing transcriptome-wide analysis to identify m⁶A-modified sites on specific RNAs. However, such approaches, or using indiscriminate chemical reagents to demethylate the whole transcriptome, alter all m⁶A modification within the transcriptome, resulting in unpredictable consequences, and making it difficult to study the effect of specific m⁶A RNA modifications.

The continually expanding CRISPR toolbox provides seemingly unprecedented opportunities for m⁶A research. At present, tool kits exists for animals (Walton et al., 2020), plants (Lu et al., 2020), and micro-organisms (Bruder et al., 2016; Westbrook et al., 2016). They have been developed rapidly, and are able to quickly and flexibly manipulate genomes in either targeted or large-scale experiments. Simultaneously, an epigenetic modification editing tool based on CRISPR has been developed. For example, fusion of dCas9 (Liu et al., 2019b) or dCas13 (Li et al., 2020b; Mo et al., 2020; Wilson et al., 2020) with the m⁶A modification enzymes, and editing the specific m⁶A site guided by single guide RNA (sgRNA) and the m⁶A protospacer adjacent motif (PAM) Locus. The m⁶A modification editing tool represents a revolutionary advance in m⁶A functional research, which will greatly enhance experimental design. Future research on m⁶A modification will focus on editing accurately m⁶A RNA modification using m⁶A editing tools, after accurately identifying m⁶A modification sites, and then functional studies at these specific m⁶A sites.

12. Final remarks and outlook

Many strategies have been devised for m⁶A identification, providing powerful tools to aid study of their biological functions. Yet, adoption of such new methods is seldom immediate, resulting in a significant lag between their development and their practical application. This may be due to the lower cost, more consistent results, greater familiarity, and

Table 1Comparison list of the m⁶A predictor Web servers.

Name	SRAMP	RAM-NPPS	BERMP	WHISTLE	WITMSG
Technologies	•Prediction based on RNA sequence	•Prediction based on RNA sequence	•Prediction based on RNA sequence	•Prediction based on RNA sequence	•Prediction based on RNA sequence
Usage	•Input genomic sequence (with introns) or cDNA/mRNA sequence (without introns).	•Input RNA sequences with FASTA format.	•Input genomic/mRNA sequences with FASTA format.	•Search by gene or biological function	•Download forecast results directly from the website.
Species coverage	•Human •Mouse	•Human <i>Saccharomyces cerevisiae</i> <i>Arabidopsis thaliana</i>	•Mammalian <i>Saccharomyces cerevisiae</i> <i>Arabidopsis thaliana</i>	•Human	•Human
Advantages	•SRAMP provides two prediction modes available: 1. Full transcript mode. 2. Mature mRNA mode. •SRAMP can provide analysis of RNA secondary structure, and provide the text representation and graphical visualization of the local RNA secondary structure of the m ⁶ A site.	•RAM-NPP is an across-species classifier for identifying m ⁶ A sites.	•BERMP is an across-species classifier for identifying m ⁶ A sites. •BERMP has more data training sets than RAM-NPP. •BERMP provides two prediction modes. 1. Full transcript mode. 2. Mature mRNA mode. (Both modes were employed for mammalian, whereas the mature mRNA mode only was used for <i>Saccharomyces cerevisiae</i> and <i>Arabidopsis thaliana</i> .)	•WHISTLE is constructed based on hundreds of transcriptome high-throughput sequencing samples, so the accuracy of m ⁶ A locus prediction has been greatly improved. •WHISTLE supports queries that may be a methylation site, a gene or a specific biological function under the Gene Ontology framework.	•WITMSG provides prediction results of m ⁶ A RNA sites on human intronic within the transcriptome.
URL	http://www.cuilab.cn/sramp	http://server.malab.cn/RAM-NPPS/	http://www.bioinfogo.org/bermp	http://whistle-epitranscriptome.com	http://rnAMD.com/intro/
Reference	Zhou et al. (2016)	Xing et al. (2017)	Huang et al. (2018a)	Chen et al. (2019a)	Liu et al. (2020)

Table 2Comparison list of the m⁶A databases.

Name	RMBase v2.0	m6Avar	m6Acomet	m6A2target	RMVar
Technologies	•miCLIP •m ⁶ A-CLIP •MeRIP-Seq	•miCLIP •PA-m ⁶ A-Seq •MeRIP-Seq •Software prediction	•miCLIP •MeRIP-Seq	•CHIP-seq •CLIP-seq •RIP-seq •MeRIP-Seq, ect.	•miCLIP •PA-m ⁶ A-Seq •DART-Seq •m ⁶ A-REF-Seq/MAZTER-seq •m6ACE-Seq •MeRIP-Seq •Search by RsID, Gene, chromosome region or disease.
Usage	•Search by Genomic Regions (BED6 Format).	•Search by RsID, Gene, chromosome region or disease.	•Search by Gene or biological function.	•Search by Gene symbol	•Search by RsID, Gene, chromosome region or disease.
Species coverage	•Human •Mouse, ect. (13 species)	•Human •Mouse	•Human	•Human •Mouse	•Human •Mouse
Advantages	•In addition to m ⁶ A modification, RMBase v2.0 also includes other modification types. •RMBase v2.0 is associated with disease-related SNV data and establishes a disease link between RNA modification sites and SNV sites. Researchers can explore the relationship between RNA modification and disease.	•m6Avar provides some information on m ⁶ A-associated variants, researchers can explore the relationship between m ⁶ A function and disease-related variants.	•m6Acomet supports the query with respect to a biological function or a number of co-methylated RNA methylation sites.	•m6A2Target is a comprehensive database for the target gene of three types of m ⁶ A related enzymes (writers, erasers and readers).	•RMVar is the updated version of m6Avar. Compared with m6Avar, RMVar contains data generated by the latest m ⁶ A detection methods (such as DART-Seq, m6A-REF-Seq, and MAZTER-seq) in the last two years, so it has more comprehensive m ⁶ A site data.
URL	http://rna.sysu.edu.cn/rmbase/	http://m6avar.renlab.org/	http://www.xjtu.edu.cn/biologicalsciences/m6acomet	http://m6a2target.canceromics.org	http://rmvar.renlab.org
Reference	Sun et al. (2015); Xuan et al. (2017)	Zheng et al. (2017)	Chen et al. (2019b)	Deng et al. (2020)	Luo et al. (2020)

the greater standardization inherent within traditional methods. We reviewed the main m⁶A detection methods currently available, comparing their advantages and disadvantages. This review should help newcomers to choose appropriate entry points and suitable research methods.

Current detection methods still require large sample sizes, lacking the necessary sensitivity to detect RNA modifications in rare and precious samples. It is also not possible to quantify accurately the number of modifications in a transcriptome, nor to identify multiple modifications simultaneously in a single experiment. Therefore, depending on the purpose of an experiment, we encourage use of new, easy-to-use, antibody-independent methods to detect m⁶A sites, improve

detection resolution, and provide accurate stoichiometries. In particular, charting single-base resolution maps of m⁶A modification will enable a more complete understanding of the biological functions of specific mRNA modification sites. This will help promote the use of CRISPR-based gene editing systems to study m⁶A-mediated functions and to edit directly specific m⁶A mRNA modification sites, while exploring their biological functions (Liu et al., 2019a; Wei and He, 2019; Wilson et al., 2020; Zheng et al., 2020).

m⁶A modification is currently the most widely studied type of RNA modification. In this review, we have described various state-of-the-art approaches to research on RNA modification, providing a handy reference to the general study of other RNA modifications.

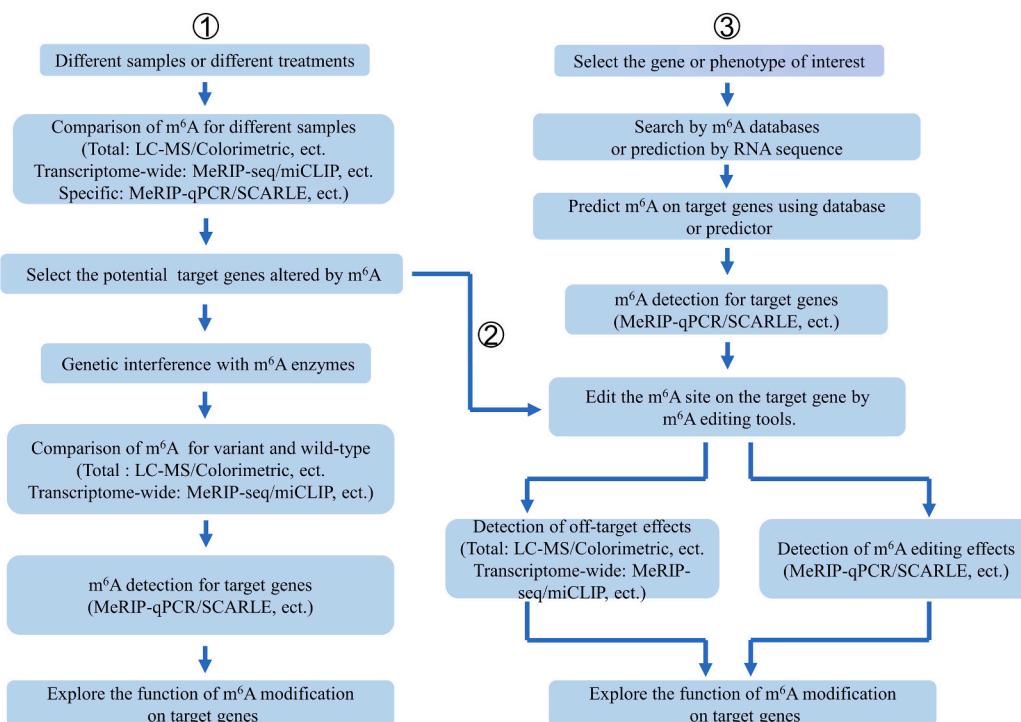
Table 3Comprehensive comparison between the different m⁶A detection methods.

Technique	Starting material	Main technique	Resolution	Time required	Initial quantity	RNA type	Stoichiometric information	Sensitivity	Reference
<i>Total m⁶A detection methods</i>									
LC-MS/MS	Any sample	LC-MS/MS	N/A	Medium	1–2 µg total RNA	mRNA total RNA	Stoichiometric	High	Thüring et al. (2017)
m ⁶ A dot blot	Any sample	Dot blot	N/A	Short	≥20 µg total RNA	mRNA	Semi-stoichiometric	Medium	Shen et al. (2016); Shen et al. (2017)
Colorimetric	Any sample	IP by m ⁶ A antibody Colorimetric	N/A	Short	100 ng–300 ng total RNA	mRNA total RNA	Stoichiometric	High	Li et al. (2020a)
<i>Specific m⁶A detection methods</i>									
meRIP-qPCR	Any sample	IP by m ⁶ A antibody RT-qPCR	100–200 nt	Short	250 ng poly(A) ⁺ RNA	poly(A) ⁺ RNA	Semi-stoichiometric	Medium	Wang et al. (2014a)
2D-TLC	Any sample	Radiolabeling RNase T1 digestion 2D-TLC	1 nt (Only GAC motif)	Long	50 ng poly(A) ⁺ RNA	poly(A) ⁺ RNA	Stoichiometric (Only GAC motif)	High	Bodi and Fray (2017)
SCARLET	Any sample	Radiolabeling RNase T1/A digestion TLC	1 nt	Long	1 µg poly(A) ⁺ RNA	poly(A) ⁺ RNA	Stoichiometric	High	Liu and Pan (2015); Liu et al. (2013)
<i>Specific m⁶A detection methods</i>									
4SedTTP-RT	Synthetic RNA	m ⁶ A sensing RT by 4SedTTP FTO digestion RT-qPCR or NGS	1 nt	Short	50 ng poly(A) ⁺ RNA	Synthetic RNA	Stoichiometric	Low	Harcourt et al. (2013)
RT-KTQ polymerase	Any sample	m ⁶ A sensing RT by RT/KTQ polymerase RT-qPCR or NGS	1 nt	Short	500 ng poly(A) ⁺ RNA or E. coli tRNA	mRNA lncRNA rRNA	Stoichiometric	Low	Aschenbrenner et al. (2018)
T3/T4 DNA ligase-qPCR	Any sample	T3/T4 DNA ligase RT-qPCR	1 nt	Short	200 nM poly(A) ⁺ RNA	poly(A) ⁺ RNA	Stoichiometry	Medium	Liu et al. (2018)
SELECT	Any sample	Bst DNA polymerase Ligation reaction RT-qPCR	1 nt	Short	3 µg total RNA	mRNA lncRNA rRNA	Stoichiometry	High	Xiao et al. (2018)
m ⁶ A melting-qPCR	Any sample	Bst DNA polymerase RT-qPCR	100–200 nt	Short	70 ng–150 ng total RNA	mRNA lncRNA 18S rRNA	Semi-stoichiometric	Low	Castellanos-Rubio et al. (2019)
<i>Transcriptome-wide m⁶A detection methods</i>									
meRIP-seq/ m ⁶ A-seq	Any sample	IP by m ⁶ A antibody NGS	100–200 nt	Long	>5 µg mRNA or >300 µg total RNA	mRNA lncRNA	N/A	Medium	Dominissini et al. (2013); Dominissini et al. (2012); Meyer et al. (2012)
<i>Transcriptome-wide m⁶A detection methods</i>									
PA-m ⁶ A-seq	Cells pre-cultured in 4SU medium	IP by m ⁶ A antibody Photo-crosslinking NGS	≥23 nt	Long	10 µg poly(A) ⁺ RNA	poly(A) ⁺ RNA	N/A	Medium	Chen et al. (2015)
m ⁶ A-CLIP/IP	Any sample	IP by m ⁶ A antibody Photo-crosslinking NGS	1 nt	Long	3 µg poly(A) ⁺ RNA	poly(A) ⁺ RNA	N/A	High	Ke et al. (2015)
miCLIP	Any sample	IP by m ⁶ A antibody Photo-crosslinking NGS	1 nt	Long	20 µg poly(A) ⁺ RNA	poly(A) ⁺ RNA	N/A	High	Hawley and Jaffrey (2019); Linder et al. (2015)
m ⁶ A-LAIC-seq	Any sample	IP by m ⁶ A antibody NGS	1500 nt	Long	150 µg total RNA	poly(A) ⁺ RNA	Semi-stoichiometric	Medium	Molinie et al. (2016)
MAZTER-seq	Any sample	MazF digestion RT-qPCR or NGS	1 nt (Only ACA motif)	Medium	100 ng poly(A) ⁺ RNA	poly(A) ⁺ RNA	Stoichiometric (Only ACA motif)	High	Garcia-Campos et al. (2019)
m ⁶ A-REF-seq	Any sample			Medium		mRNA		High	Zhang et al. (2019)

(continued on next page)

Table 3 (continued)

Technique	Starting material	Main technique	Resolution	Time required	Initial quantity	RNA type	Stoichiometric information	Sensitivity	Reference
<i>Transcriptome-wide m⁶A detection methods</i>									
DART-seq	APOBEC1-YTH expression cells	MazF digestion FTO digestion NGS	1 nt (Only ACA motif)	Long	100 ng-200 ng mRNA	ploy (A) ⁺ RNA	Stoichiometric (Only ACA motif)	Low	Meyer (2019)
Metabolic propargyl labeling									
propargyl labeling	Cells pre-cultured in propargyl-L-selenomethionine medium	Gene editing Cell transfection NGS	1-2 nt	Long	0.25-0.75 µg total RNA	mRNA	Stoichiometric	Medium	Hartstock et al. (2018)
m ⁶ A-label-seq	Cells pre-cultured in allyl-SeAM medium	Metabolite labeling NGS	1 nt	Long	5 µg a ⁶ A-modified mRNAs	mRNA	N/A	Medium	Shu et al. (2020)
SMRT	Synthetic RNA	SMRT	1 nt	Medium	N/A	Synthetic RNA	N/A	Low	Vilfan et al. (2013)
Nanopore DRS	Any sample	Nanopore DRS	1 nt	Medium	1 µg ploy (A) ⁺ RNA	ploy (A) ⁺ RNA	N/A	Low	Ayub and Bayley (2012); Liu et al., 2019a; Parker et al. (2020)

**Fig. 9.** Design and analysis of m⁶A experiments.**Author contributions**

Hong-xiang Zheng and Na Sui prepared the manuscript. Na Sui and Xian-sheng Zhang conceptualized the idea and revised the manuscript. All authors read and approved the final manuscript.

Declaration of Competing interests

The authors declare no competing interests.

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