RNA Methylation

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Engineering of a DNA Polymerase for Direct m^{6}A Sequencing

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Abstract: *Methods for the detection of RNA modifications are of fundamental importance for advancing epitranscriptomics. N^6-methyladenosine (m^{6}A) is the most abundant RNA modification in mammalian mRNA and is involved in the regulation of gene expression. Current detection techniques are laborious and rely on antibody-based enrichment of m^{6}A-containing RNA prior to sequencing, since m^{6}A modifications are generally "erased" during reverse transcription (RT). To overcome the drawbacks associated with indirect detection, we aimed to generate novel DNA polymerase variants for direct m^{6}A sequencing. Therefore, we developed a screen to evolve an RT-active KlenTaq DNA polymerase variant that sets a mark for N^6-methylation. We identified a mutant that exhibits increased misincorporation opposite m^{6}A compared to unmodified A. Application of the generated DNA polymerase in next-generation sequencing allowed the identification of m^{6}A sites directly from the sequencing data of untreated RNA samples.*

Cellular RNAs are posttranscriptionally modified through the enzymatic introduction of more than 150 modifications.[1] The research field of epitranscriptomics aims to investigate the role of these modifications, which possess functional importance but do not alter the RNA sequence itself.[2] Therefore, reliable and straightforward methods to detect modifications in a transcriptome-wide manner are required. However, while nucleic acid analysis in general has profited tremendously from the rise of next-generation sequencing (NGS) technologies[3],the enormous potential of these techniques has so far only rarely been adapted for the direct analysis of modified nucleotides. This is because modifications in an RNA template strand that do not alter the sequence are "erased" during reverse transcription (RT). Modifications located at the Watson–Crick face of the nucleobase constitute an exception to this rule since they affect RT, resulting in the appearance of "RT signatures" at modification sites.[4] These signatures arise from increased incorporation of mismatched nucleotides and/or accumulated rates of RT abortion at modification sites. On this basis, direct prediction of N^1-methyladenosine (m^{1}A) sites from NGS sequencing data has been realized.[5] This approach is, however, restricted to modifications that interfere with correct Watson–Crick base pairing. To overcome this limitation, we aimed to evolve a novel RT system that introduces signatures opposite a normally RT-silent modification.

N^6-methyladenosine (m^{6}A) was chosen as target modification because it is a reversible[6] and highly abundant[7] modification in mammalian mRNA. m^{6}A modification of cellular RNA has been demonstrated to affect mRNA splicing,[8] nuclear export,[6b,9] translation,[10] and degradation.[11] Proposed functions include the generation of "translational pulses",[12] the control of the circadian clock,[9] the initiation of the DNA damage response,[13] and the clearance of maternal RNA[14] and pluripotency factors.[15] Furthermore, m^{6}A modification can also be found in other cellular RNAs, including rRNA, tRNA, and lncRNA.[1a,2c] Current methods to map m^{6}A typically employ immunoprecipitation of m^{6}A-specific antibodies and covalent crosslinking of the antibody to the RNA molecule prior to analysis by NGS.[7a,b,16] These methods are complex and laborious and the results may suffer from artifacts deriving from poor antibody specificity and cross-reactivity.[17] Therefore, novel m^{6}A detection systems might benefit from reverse transcriptases (RTases) that sense m^{6}A during cDNA synthesis and create a signal that can be passed on during PCR. The fact that certain RT-active DNA polymerases are capable of discriminating m^{6}A from unmodified A has been shown by a previous study.[18] We were able to advance this feature by engineering an RT-active DNA polymerase that features significantly increased error rates opposite m^{6}A but not unmodified A. The enzyme was evolved from a thermostable KlenTaq DNA polymerase variant with RT activity (KlenTaq L459M S515R I638F M747K, henceforth referred to as RT-KTQ).[19]

As a first step, the incorporation of complementary and non-complementary nucleotides opposite m^{6}A and unmodified A by RT-KTQ was investigated. Single-nucleotide incorporation was performed with each of the four dNTPs, employing a primer hybridized to two different RNA oligonucleotides of the same sequence carrying either A or m^{6}A at the site of first incorporation (Figure S1 in the

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Supporting Information). As expected, misincorporation of dAMP, dCMP, and dGMP was considerably less efficient than incorporation of the complementary dTMP for both templates. Moreover, dTMP and dAMP incorporation differed only slightly between the A and m^6A templates, whereas dCMP and dGMP incorporation was significantly hampered opposite m^6A. Inspired by previous studies that utilized capillary electrophoresis (CE) to monitor the activity of DNA polymerases and other enzymes,[20] we conceived an assay to screen for DNA polymerase variants with increased misincorporation opposite m^6A. The screen involved extension of 5′-fluorophore-labeled primer strands through single-nucleotide incorporation, followed by CE. Multiplexed analysis of several primer extension reactions could be achieved by employing primers of different lengths labeled with different fluorophores (Figure 1 a). More specifically, six primers were designed that possess the same 3′-terminal 20 nucleotides complementary to the RNA template but differ in their length due to varying 5′-overhangs (Table S1 in the Supporting Information). Additionally, primers were employed as 5′-FAM- and 5′-HEX-labeled variants. The devised assay was applied to screen a library composed of RT-KTQ single mutants created by site-directed mutagenesis. Mutation sites were selected based on their vicinity to the nascent base pair in a crystal structure of an RT-KTQ closed complex[19a] (Figure 1 b). For each site, all 19 mutants were generated and expressed in *E.coli* in 96-well plates. Single-nucleotide incorporation was then performed with the RT-KTQ expression lysates after heat-denaturation of the *E.coli* host proteins.[21]

It was reported that low-fidelity DNA polymerases differ from high-fidelity DNA polymerases mainly in the efficiency of correct nucleotide incorporation, whereas the incorporation of incorrect nucleotides is comparable.[22] Thus, we reasoned that enhanced error rates opposite m^6A would probably derive from decreased dTMP incorporation rather than from increased misincorporation. For this reason, the developed screening assay was employed to monitor dTMP incorporation opposite m^6A and unmodified A. Here, we looked for variants with considerably decreased incorporation of dTMP opposite m^6A but not A (Figure S2). Furthermore, to ensure that only incorporation of the correct nucleotide was reduced and not overall activity, dAMP incorporation was monitored in an additional screening (Figure S3). Evaluation of the screening data was performed by qualitative assessment of extension peaks in the electro-

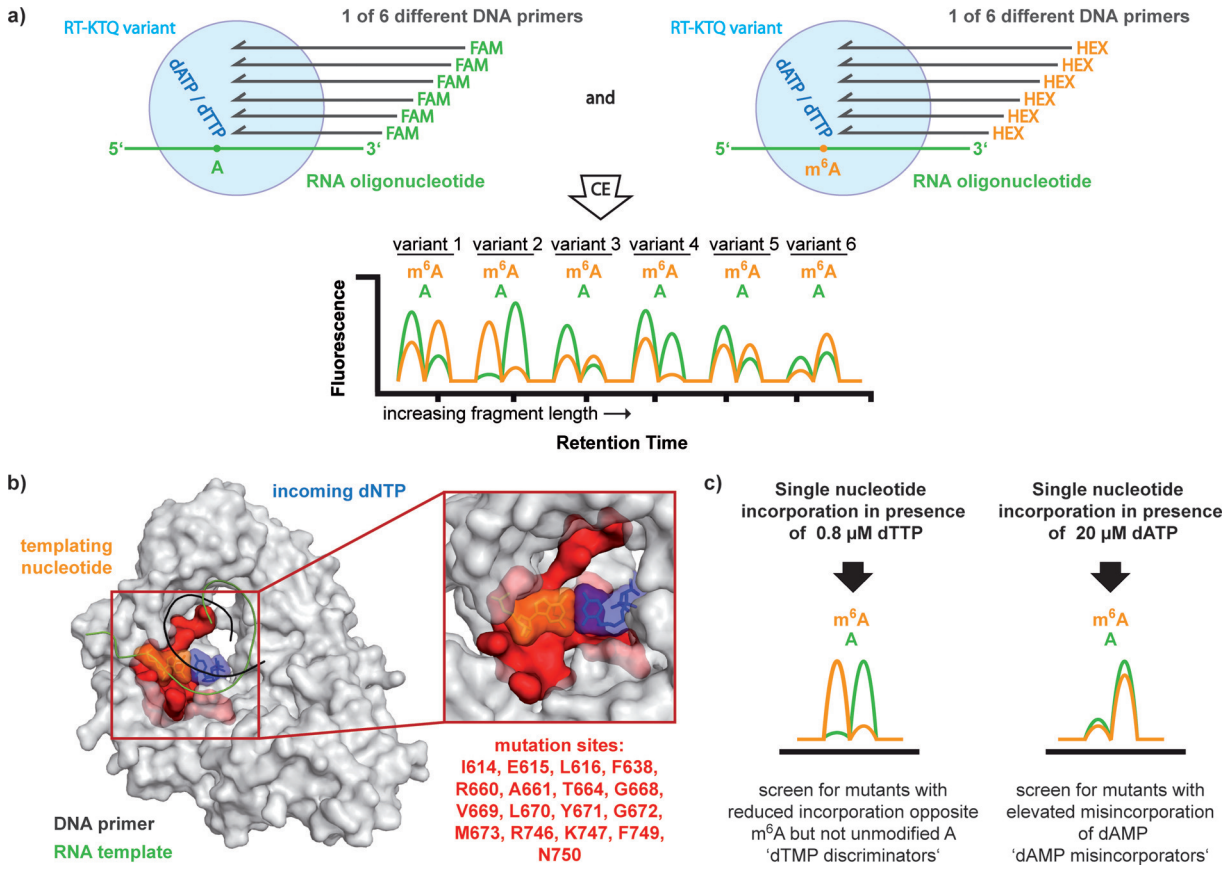

Figure 1. Screening for DNA polymerase variants with increased misincorporation rates opposite m^6A. a) DNA polymerase expression lysates were applied to catalyze the incorporation of dTMP or dAMP opposite A and m^6A. Utilization of primers with different length and fluorophores (FAM = 6-carboxyfluorescein; HEX = hexachlorofluorescein) enabled the joint analysis of 12 reaction mixtures in one capillary. b) Amino acids in proximity to the nascent base pair were chosen for saturation mutagenesis. Adapted from PDB ID: 4BWM[19a] using PyMOL (Schrödinger, LLC; New York, NY). c) Anticipated outcome for promising RT-KTQ variants: high m^6A discrimination for dTMP incorporation and high efficiency for dAMP misincorporation.

pherogram (Figure 1c). Significantly increased "dTMP discrimination" was achieved by many RT-KTQ variants with mutations of G672, G668, Y671, or M673 and by some sporadic variants with mutations at other positions (Figure S2). Many variants with mutations of I614, A661, T664, G668, and Y671 featured comparatively high dAMP misincorporation (Figure S3). Mutations with the most prominent effect on m^{6}A discrimination were combined with mutations exerting the greatest effect on dAMP misincorporation to create a second-generation library containing all possible double mutants with one "discriminator" mutation (L616T, Y671A, G672H, G672A, G672K, M673T, R746K) and one "misincorporator" mutation (I614A, A661K, T664K, G668Y, Y671T, F749P). This library was screened in the same manner (Figure S4) and the most promising mutants from both libraries were affinity purified followed by evaluation of their error rates at m^{6}A sites.

For this purpose, the selected RT-KTQ variants were applied for the RT step in a previously published NGS library preparation method that includes RT-stop products within the PCR amplified library.[5] As a template, we employed the m^{6}A-containing RNA oligonucleotide used in the initial screening. After sequencing and data processing, sequences were mapped to the reference sequence and error-rate signatures were extracted and visualized by employing CoverageAnalyzer.[23] While most of the RT-KTQ variants exhibited regular error rates at the m^{6}A site, rates were considerably elevated for variants carrying mutations at amino acid Y671 (Table S2, Figure S5). Two single mutants (Y671A and Y671T) and one double mutant (G668Y Y671A) featured particularly prominent signatures. The highest overall error rate of about 15% was measured for RT-KTQ G668Y Y671A (Figure 2). Here, 0.1% G-reads (due to dCMP incorporation during cDNA synthesis), 10% T-reads (dAMP incorporation), and 4.7% C-reads (dGMP incorporation) were present at the modification site. Moreover, when looking at the overall sequencing profile for this enzyme, the m^{6}A site was the only site with an error rate of more than 10%, and error rates did not exceed 5% for any of the unmodified adenosines in the template (Figure 2). Interestingly, the engineered DNA polymerases tend to stall after the misincorporation of non-complementary nucleotides opposite m^{6}A, resulting in cDNA termination directly adjacent to the modification site. Thus, the measured elevated error rates will only be observable when RT-stop products are included within the library.

RT-KTQ G668Y Y671A was further employed for the analysis of a known m^{6}A site in *E.coli* tRNA Val.[1a] We employed isolated *E.coli* tRNA extracts as a template for library preparation. Once again, a significantly elevated error rate was observed at the m^{6}A site (14.3%; Figure 3). The only other sites with error rates of more than 10% were located opposite another modified nucleotide (5-methyluridine, T) or at the 5′-end of the RNA molecule, where rates are inaccurate due to low coverage. The lower coverage derives from reduced activity of the enzyme (Table S3) and synthesis arrest on account of tRNA secondary structure and modifications, and it cannot be resolved decisively by altered reaction conditions.[5] Another modification that affected RT

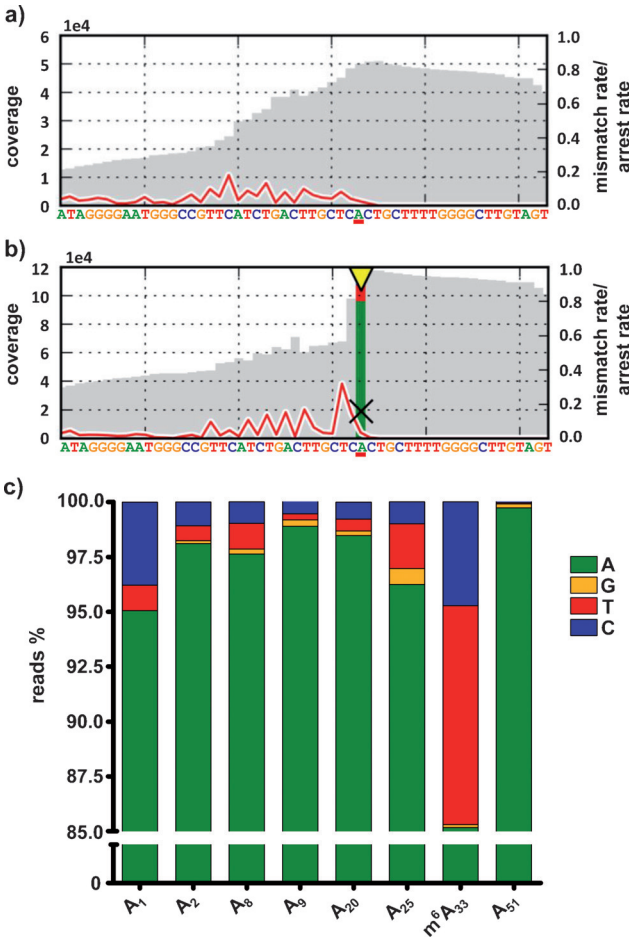

Figure 2. RT-KTQ G668Y Y671A features elevated error rates opposite m^{6}A. a,b) Sequencing profiles of an m^{6}A-containing RNA oligonucleotide reverse transcribed by unmodified RT-KTQ (a) and RT-KTQ G668Y Y671A (b). Sites with error rates of more than 10% are highlighted with yellow arrows, with colored bars indicating the nature of the reads. Mismatch rates are depicted as black crosses, arrest rates as red lines. The m^{6}A site is indicated with a red underline. Figure created with CoverageAnalyzer.[23] C) Mismatch signature of RT-KTQ G668Y Y671A opposite m^{6}A and all unmodified As present in the RNA oligonucleotide.

by this enzyme was uridine-5-oxyacetic acid (V), which triggered high rates of RT arrest. In contrast, almost all unmodified A sites exhibited error rates below 5%. Only A_{35} constituted an exception, with an error rate of 8.5%. We assume that the increased error rate at this position might arise from the fact that this nucleotide is located directly adjacent to the RT-affecting uridine-5-oxyacetic acid. The m^{6}A signature was not observed when unmodified RT-KTQ was applied (Figure S6). When comparing the two analyzed m^{6}A sites, misincorporation and arrest patterns vary due to different sequence contexts, as has been observed previously for m^{1}A signatures.[5]

We aimed to investigate why the engineered RT-KTQ G668Y Y671A double mutant exhibited an elevated error rate opposite m^{6}A (and an increased amount of T-reads in particular), whereas other mutations identified by the initial screening did not show this effect. Therefore, we determined

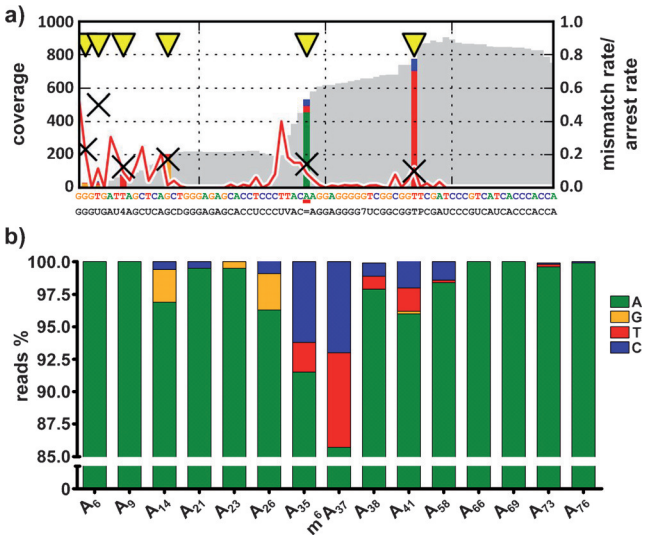

Figure 3. Analysis of a known m^{6}A site in *E.coli* tRNA Val b applying RT-KTQ G668Y Y671A. a) Sequencing profile of *E.coli* tRNA Val reverse transcribed by RT-KTQ G668Y Y671A. Sites with error rates of more than 10% are highlighted with yellow arrows, with colored bars indicating the nature of the reads. Mismatch rates are depicted as black crosses, arrest rates as red lines. The colored sequence at the top represents the expected cDNA sequence. The black sequence at the bottom is the actual sequence of tRNA Val containing all its modified nucleotides ('4′ = 4-thiouridine; 'D′ = dihydrouridine; 'V′ = uridine-5-oxyacetic acid; ′ = ′ = m^{6}A; '7′ = 7-methylguanosine; 'T′ = 5-methyluridine; 'P′ = pseudouridine).[1a] Figure created with CoverageAnalyzer.[23] b) Mismatch signature of RT-KTQ G668Y Y671A opposite m^{6}A and all unmodified As present in *E.coli* tRNA Val.

the incorporation rates of dAMP and dTMP opposite A and m^{6}A at a given dNTP concentration of 100 μM (Table S3, Figure S7, S8). For unmodified RT-KTQ, the ratio of dTMP to dAMP incorporation rate was similar for A and m^{6}A. For RT-KTQ Y671A, dAMP misincorporation rates were comparable to the unmodified RT-KTQ. However, dTMP incorporation was significantly reduced opposite m^{6}A, whereas it decreased only slightly opposite A. A similar effect was observed for the G668Y mutation. It was necessary to combine both mutations in an RT-KTQ double mutant to attain an increased amount of T-reads at the m^{6}A site. Correspondingly, RT-KTQ G668Y Y671A featured an even further reduced incorporation rate of dTMP opposite m^{6}A. For this enzyme, dTMP incorporation opposite m^{6}A was only 1.6 times faster than dAMP misincorporation. In contrast, mutation of residues I614 and G672 did not result in elevated error rates opposite m^{6}A. Whereas the G672H mutation delivered the most prominent discrimination of m^{6}A during dTMP incorporation, it also hampered the misincorporation of dAMP tremendously. RT-KTQ I614A featured significantly increased rates of dAMP misincorporation but lost m^{6}A discrimination.

In this study we provide a novel engineering strategy to create reverse transcriptase variants exhibiting RT signatures as a response to encountering a specific RNA modification. The strategy to evolve an "m^{6}A-sensing" RT-active DNA polymerase involved the generation of DNA polymerase libraries in combination with a primer-extension-based screening assay. Notably, the assay should also be suitable for other modifications and the throughput of the assay could be increased for future projects by employing more primers of different length, a greater variety of 5′-fluorophores, and/or several orthogonal primer/template sequences. Qualitative examination of the screening data for variants with increased m^{6}A discrimination during dTMP incorporation but with unaffected dAMP misincorporation delivered promising mutants. Interestingly, the identified Y671 residue is located directly at the C-terminal end of the O-helix and is known to undergo substantial conformational changes upon dNTP binding, thereby playing an important role in the selectivity of KlenTaq DNA polymerase and the homologous KF DNA polymerase.[24]

We have been able to show that the engineered RT-KTQ G668Y Y671A delivers prominent RT signatures at m^{6}A sites in different sequence contexts, without exerting elevated error rates opposite unmodified nucleotides and the majority of the other modified nucleotides present in the *E.coli* tRNA Val. Only uridine-5-oxyacetic acid and 5-methyluridine resulted in the emergence of high arrest rates and increased dGMP misincorporation, respectively. However, these RT signatures are highly characteristic, which might enable their distinction from (and detection simultaneously to) m^{6}A. RT-KTQ G668Y Y671A could contribute to the development of new sequencing approaches to map m^{6}A sites in cellular RNA in the future. Here, a technology that is orthogonal to the present antibody-enrichment methodologies (MeRIP)[7a,b,16] is desperately needed to validate candidate sites[17] and to simplify detection procedures. The development of such assays necessitates algorithms to identify m^{6}A-specific RT signatures and to distinguish them from signals deriving from other sources. As already implemented for the detection of m^{1}A, machine-learning-based algorithms can be trained to predict modification sites when fed with sufficient data for modification-specific RT signatures.[5] For this purpose, sequencing data from modified RNA oligonucleotides and/or validated m^{6}A sites in rRNA, mRNA, or lncRNA could be utilized to generate training data sets.[18,25]

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Conflict of interest

The authors declare no conflict of interest.

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