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Supporting Information

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

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Table of Contents

Methods

Oligonucleotides and *E.coli* tRNA. DNA oligonucleotides were purchased HPLC purified from Biomers and IBA Lifesciences and directly applied for Illumina library preparation and primer extension reactions analyzed by capillary electrophoresis. For adapter ligation, the 3'-adapter DNA oligonucleotide was preadenylated as described.[1] For primer extension assays with radioactively labeled primers, oligonucleotides were further purified by preparative PAGE prior to labeling with [γ-^{32}P]-ATP and T4 polynucleotide kinase (New England Biolabs) according to the vendor's protocol. RNA oligonucleotides were purchased PAGE purified from Purimex. The sequences of all used oligonucleotides are listed in Table S1. Purified *E.coli* tRNA was prepared as described.[1]

Protein expression and purification. Protein expression was performed in *E.coli* BL21 DE3 (Novagen) as described.[2] Purification of 6x His-tagged KlenTaq DNA polymerase variants was achieved via heat denaturation of lysates at 75°C for 45 min, followed by ultracentrifugation at 20.000 rpm for 45 min. Then 5 mM imidazole was added and affinity purification was performed employing the cOmplete™ His-Tag purification Resin (Roche). Therefore, 1 ml beads were washed 3 times with 5 ml calibration buffer (5 mM imidazole, 200 mM NaCl, 2.5 mM $MgCl_2$, 0.1% (v/v) Triton X-100, 10 mM Trizma® (pH 9.2)) by centrifugation and then resuspended in 0.5 mL calibration buffer and added to the lysates. After shaking for 3 h at 4°C, the supernatant was discarded and 12 mL washing buffer I was added (10 mM imidazole, 200 mM NaCl, 2.5 mM $MgCl_2$, 0.1% (v/v) Triton X-100, 10 mM Trizma® (pH 9.2)) to the beads. Beads were washed at 4°C for 5 min, followed by centrifugation and another washing step with 12 mL washing buffer II (15 mM imidazole, 200 mM NaCl, 2.5 mM $MgCl_2$, 0.1% (v/v) Triton X-100, 10 mM Trizma® (pH 9.2)). Subsequently, elution was achieved by addition of 3 mL elution buffer I (100 mM imidazole, 5 mM $MgCl_2$, 100 mM Trizma® (pH 9.2)) and shaking at 4°C for 20 min. Enzymes were then concentrated by Vivaspin (Sartorius), washed 3 times with 20 mL elution buffer II (5 mM $MgCl_2$, 100 mM Trizma® (pH 9.2)) to get rid of the imidazole and stored in storage buffer (50 mM Trizma® (pH 9.2), 2.5 mM $MgCl_2$, 16 mM $(NH_4)_2SO_4$, 0.1% (v/v) Tween 20, 50% (v/v) Glycerol) at −20°C. Purity of enzymes was validated by SDS-PAGE.

RT-KTQ library construction. All possible RT-KTQ single mutants at the positions I614, E615, L616, F638, R660, A661, T664, G668, V669, L670, Y671, G672, M673, R746, K747, F749 and N750 were created by site directed mutagenesis of the respective codons as described.[2b] 19 separate PCR reactions were performed for each site respectively, each with the same 5'-phosphorylated reverse primer, but with an individual forward primer carrying the triplet coding for the destined amino acid. After PCR amplification employing Phusion® DNA polymerase (New England Biolabs) and DpnI (New England Biolabs) digestion of the template plasmid, reactions were purified using a NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel). PCR products were ligated using T4 DNA ligase (New England Biolabs) and transformed into *E.coli* BL21 DE3 (Novagen). Plasmids were sequenced by Sanger sequencing (GATC Biotech) and clones carrying plasmids with correct RT-KTQ mutants were cultured overnight in 700 µl LB-medium containing 100 µg/ml carbenicillin in 96-well deep-well plates at 37°C. Subsequently, 700 µl of 60% (v/v) glycerol in LB-medium was added and plates were stored at -80°C. RT-KTQ double mutants derived from site directed mutagenesis employing the plasmids carrying the genes of RT-KTQ single mutants as template.

Screening for RT-KTQ mutants with elevated error rates opposite m^{6}A but not unmodified A. RT-KTQ variants were expressed in 96 well plates and lysed in KlenTaq reaction buffer (50 mM Trizma® (pH 9.2), 16 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 0.1% (v/v) Tween 20) containing 0.5 mg/ml

lysozyme by incubation at 37°C for 20 min. After denaturation of *E.coli* host proteins by incubation at 75°C for 45 min, plates were centrifuged at 4400 rpm and 4°C for 30 min and lysates were directly deployed for single nucleotide incorporation. First, 4 µL lysate per well were transferred to a fresh 96 well reaction plate (on ice) applying a multichannel pipette. Here, only half of the plate was transferred twice (i.e. either column 1-6 or column 7-12) to obtain duplicates of each enzyme in one reaction plate (i.e. in the new plate columns 1-6 and columns 7-12 were identical). Plates were heated to 55°C on a thermocycler and 16 µl of reaction mixture (1.6 µL of (10x) KlenTaq reaction buffer, 2 µl of 200 µM dATP or 8 µM dTTP, 2 µL of annealed primer-template (50 nM primer / 100 nM template), and 10.4 µL Milli-Q) were added from ice to start the reaction. 12 reactions could be started at once when applying a multichannel pipette. The reaction mix for columns 1-6 contained RNA template A, the mix for columns 7-12 contained RNA template m6A (Table S1). Primers differed for each column: (1) 20 nt FAM, (2) 25 nt FAM, (3) 30 nt FAM, (4) 35 nt FAM, (5) 40 nt FAM, (6) 45 nt FAM, (7) 20 nt HEX, (8) 25 nt HEX, (9) 30 nt HEX, (10) 35 nt HEX, (11) 40 nt HEX, (12) 45 nt HEX (Table S1). Annealing of an adequate amount of the respective primer and template was performed beforehand in the correct order in a 12-tube PCR strip by heating to 95°C for 2 min and subsequent incubation on ice. Then the appropriate volume of a 'mastermix' containing the remaining components (buffer, dNTP, Milli-Q) was added to each tube. This line of action facilitated the use of multichannel pipettes to transfer the correct primer extension mixture (containing the respective primer-template complex) to the correct target well. Reactions were incubated for 10 min at 55°C (during this time all rows of the 96 well plate could be started consecutively) and stopped by addition of 20 µL CE stop solution (80% (v/v) formamide, 20 mM EDTA) after 10 min consecutively. Reaction mixtures were then analyzed directly by capillary electrophoresis. For each lysate plate, reactions had to be performed in 4 reaction plates: for each half of the lysate plate one reaction plate with dATP and one with dTTP. The suitable concentrations of dATP (20 µM) and dTTP (0.8 µM) were determined in a preceding experiment by a dilution series employing lysate of unmodified RT-KTQ.

Capillary electrophoresis (CE). Primer extension reactions were directly analyzed by CE. Therefore, 38 µL of Hi-Di™ Formamide (containing 0.5% (v/v) GeneScan™ 120 LIZ™; Thermo Fisher Scientific) were added to each well of one column of a MicroAmp™ 96-well plate (Thermo Fisher Scientific). Then 1 µl of each of the 12 reactions from one row of the 96 well reaction plate was transferred to an identical well of the MicroAmp™ plate to obtain a final volume of 50 µL per well. Consecutive transfer of each column from the reaction plate to the target column in the MicroAmp™ plate by a multichannel pipette facilitated this process. CE was then performed by employing the Applied Biosystems 3500 Genetic Analyzer with an 8-capillary array (50 cm) filled with POP-6™ polymer (Thermo Fisher Scientific). The following parameters were applied for the CE run: G5 dye set, 60°C oven temperature, 1900 s run time, 13.0 kV run voltage, 180 s prerun time, 13 kV prerun voltage, 50 s injection time, 1.6 kV injection voltage, 1 s data delay. Qualitative assessment of the data was carried out applying the GeneMapper™ Software 5.

Primer extension with radioactively labeled primers. The reaction mixtures contained 150 nM of [γ-^{32}P]-labeled primer (RNAoligo primer rev (-1)), 225 nM template (RNA template A or m^{6}A) and 100 µM dNTP in KlenTaq reaction buffer (50 mM Trizma® (pH 9.2), 16 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 0.1% (v/v) Tween 20). Reaction mixtures (20 µL, respectively) were heated to 95°C for 2 min and subsequently cooled to 55°C. After starting the primer extension by addition of 1 nM (or 20 nM for mismatch reactions in Figure 1) of the RT-KTq variant, reactions were allowed to proceed for the desired reaction times (see Figure 1/S3). Reactions were quenched by the addition of 20 µl stop solution (80%

formamide, 20 mM EDTA, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol) and analyzed by 12 % denaturing PAGE. Visualization was performed by phosphorimaging.

Library preparation and sequencing. Library preparation was performed according to a previously published protocol.[1,3] Correspondingly, 500 ng of RNA template m^6A or purified *E.coli* tRNA were denatured at 90°C for 30 s, chilled on ice and dephosphorylated by the addition of 0.5 u FastAP alkaline phosphatase (Thermo Fisher Scientific) in 100 mM Tris-HCl (pH 7.4), 20 mM $MgCl_2$, 0.1 mg/mL BSA, and 100 mM 2-mercaptoethanol with a total volume of 10 µL. After incubation of the reaction mixture at 37°C for 30 min, the denaturation step was repeated and another 0.5 u of FastAP were added, followed by another incubation at 37°C for 30 min and heat inactivation at 75°C for 5 min. Then 5 µM of preadenylated 3'-adapter[1], 1 u T4 RNA ligase 2 truncated, 0.5 u T4 RNA ligase and 15% DMSO were added in a final volume of 20 µL and ligation was performed by incubation of the reaction mixture at 4°C overnight. After heat inactivation of the enzymes at 75°C for 15 min, 50 u of 5'-deadenylase (New England Biolabs) were added and the mixture was incubated at 30°C for 30 min, afterwards at 90°C for 30 s and then chilled on ice. Another portion of 5'-deadenylase was added and another round of incubation steps was performed, followed by the addition of 10 u Lambda exonuclease (Thermo Fisher Scientific) and incubation at 37°C for 30 min, 90°C for 30 s and on ice for 2 min. This step was also repeated. Then ethanol precipitation was performed and the pellet was dissolved in 20 µL RT reaction mixture containing 5 µM RT-primer in KlenTaq reaction buffer (50 mM Trizma® (pH 9.2), 16 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 0.1% (v/v) Tween 20). The mixture was incubated at 75°C for 5 min and chilled on ice, before 250 nM of the respective RT-KTQ variant and 200 µM dNTPs (each) were added and RT was performed at 60°C for 1 h. Residual primer was digested by addition of 10 u Lambda exonuclease and incubation at 37°C for 30 min, followed by addition of another portion of the enzyme and another incubation. Subsequently, 40 u of Exonuclease I (Thermo Fisher Scientific) was added and the mixture was incubated at 37° for 30 min. This step was also repeated, followed by heat denaturation at 80°C for 15 min. Residual dNTPs were dephosphorylated with 2 u FastAP alkaline phosphatase for 30 min at 37°C, followed by heat denaturation at 90°C for 2 min and cooling on ice. After repetition of the dephosphorylation step and another heat inactivation at 75°C for 5 min, RNA was degraded by the addition of NaOH to a final concentration of 0.15 M and heating to 55°C for 25 min. Reactions were cooled on ice and acetic acid was added to a final concentration of 0.15 M, followed by another ethanol precipitation. The pellet was dissolved in 10 µL tailing mixture containing 1.25 mM CTP and 10 u TdT in TdT buffer (Thermo Fisher Scientific) and tailing was carried out at 37°C for 30 min, followed by heat inactivation at 70°C for 10 min. Subsequently, 1.25 µM ds 5'-adapter (annealed 5'-adapter strand 1 and 2), 10 µM ATP, 60 u T4 DNA ligase HC (Thermo Fisher Scientific), 50 mM Tris HCl (pH 7.4), and 10 mM $MgCl_2$ were added to obtain a final volume of 40 µL and the mixture was incubated at 4°C for 18 h. After heat denaturation at 75°C for 15 min, ethanol precipitation was performed. The 40-150 nucleotide size fraction of the mixture was obtained by preparative denaturing PAGE, followed by excision of the respective bands, and elution in 0.5 M ammonium acetate. After another ethanol precipitation, PCR was performed by dissolving the pellet in 20 µl PCR reaction mixture containing 3 mM $MgCl_2$, 0.5 mM dNTP (each), 5 µM P7 PCR primer, 5 µM P5 PCR primer, and 5 u Taq DNA polymerase in Taq buffer (Rapidozym). After initial denaturation for 5 min at 95°C, amplification was achieved by 12 cycles of 1 min at 95°C, 1 min at 65°C and 1 min at 72°C, followed by a final extension of 5 min at 72°C. After ethanol precipitation, a final size fractionation step was conducted by preparative denaturing PAGE, excision of bands between 150 and 300 nt, elution in 0.5 M ammonium acetate, and ethanol precipitation. DNA concentration was determined using Nanodrop™. After quality control (Agilent 2100 Bioanalyzer) and pooling of the libraries, sequencing was performed in paired-end mode on an Illumina MiSeq instrument.

Processing of sequencing data. Processing of sequencing data was done with a custom bioinformatics pipeline. Trimming of primers, adapters, barcodes and tailing overhangs were assembled and performed in a Python-based workflow, using Cutadapt v.1.8.1 software [4]. Bowtie2 aligner[5] was used for mapping with tRNA references from MODOMICS[6] and the synthetic oligo reference. Parameters for alignment were end-to-end (global) with one mismatch (-N 1) tolerated in the seed of 6 nucleotides (-L 6). The setting was adjusted to only report one (k=1) alignment for each read under simultaneous mapping to all references. Format conversion of SAM files after mapping was performed with SAMtools utility[7], generating sorted and indexed BAM files which were translated to Pileup format. For further format conversion CoverageAnalyzer[8] was used to generate a custom tab-separated text file format, denoted profile, including all parameters (coverage, mismatch rate, arrest rate, counts for each base type) for each reference position. The presented RT-signatures were compiled with CoverageAnalyzer[8].

Supplementary Tables

Table S1. Oligonucleotides applied in this study.

Type	Oligonucleotide	Sequence
RNA	RNA template A	5'-AUAGGGGAAUGGGCCGUUCAUCUGACUUGCUC **A** CUGCUUUUG GGGCUUGUAGU -3'
RNA	RNA template m^6A	5'-AUAGGGGAAUGGGCCGUUC AUCUGACUUGCUC **m^6A** CUGCUUUUG GGGCUUGUAGU -3'
DNA	RNAoligo primer rev (-1)	5'-ACTACAAGCCCCAAAAGCAG-3'
DNA	Screening primer 20 nt HEX/FAM	5'-(F)-ACTACAAGCCCCAAAAGCAG-3'
DNA	Screening primer 25 nt HEX/FAM	5'-(F)-CGATC ACTACAAGCCCCAAAAGCAG-3'
DNA	Screening primer 30 nt HEX/FAM	5'-(F)-TCGAT CGATC ACTACAAGCCCCAAAAGCAG-3'
DNA	Screening primer 35 nt HEX/FAM	5'-(F)-ATCGA TCGAT CGATC ACTACAAGCCCCAAAAGCAG-3'
DNA	Screening primer 40 nt HEX/FAM	5'-(F)-GATCG ATCGA TCGAT CGATC ACTACAAGCCCC AAAAGCAG-3'
DNA	Screening primer 45 nt HEX/FAM	5'-(F)-CGATC GATCG ATCGA TCGAT CGATCACTACAAG CCCCAAAAGCAG-3'
DNA	3'-adapter	5'-(P)-CNNNNNNNNNAGATCGGAAGAGCGTCGTGTA GGGAAAGAGTGT-3'-C6-spacer
DNA	RT-primer	5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-3'
DNA	5'-adapter strand 1	5'-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGG-3'
DNA	5'-adapter strand 2	5'-(P)-AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-3'-C6-spacer
DNA	P7 PCR primer	5'-CAAGCAGAAGACGGCATACGAGAT77777777 GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-3'
DNA	P5 PCR primer	5'-AATGATACGGCGACCACCGAGATCTACAC55555555 ACACTCTTTCCCTACACGACGCTCTTCCGATCT-3'

(P) = phosphate. (F) = fluorophore (primers were used as HEX- and 6-FAM-labeled). N = A/C/G/T. (7777777) and (55555555) constitute standard Illumina barcodes (N701-N712 and N501-N508).

Table S2. Error rates of selected RT-KTQ variants opposite m^6A in an RNA oligonucleotide.

DNA polymerase	Overall error rate [%]	A-reads [%]	G-reads [%]	T-reads [%]	C-reads [%]
RT-KTQ	2.2	97.8	0.2	0.8	1.2
RT-KTQ I614A	2.7	97.3	0.2	1.6	1.0
RT-KTQ I614K	1.9	98.1	0.3	1.3	0.3
RT-KTQ A661K	1.8	98.2	0.2	0.9	0.7
RT-KTQ T664K	1.5	98.5	0.2	0.6	0.7
RT-KTQ G668Y	3.1	96.9	0.2	1.8	1.1
RT-KTQ Y671A	14.5	85.5	0.1	2.0	12.4
RT-KTQ Y671T	10.5	89.5	0.1	5.6	4.9
RT-KTQ G672A	2.1	97.9	0.2	1.0	0.9
RT-KTQ G672F	4.1	95.9	0.2	2.2	1.7
RT-KTQ G672H	1.7	98.3	0.2	0.8	0.7
RT-KTQ G672K	2.0	98.0	0.3	0.8	1.0
RT-KTQ G672W	3.7	96.3	0.1	1.8	1.8
RT-KTQ M673T	2.0	98.0	0.1	1.0	0.8
RT-KTQ R746K	2.0	98.0	0.2	0.7	1.0
RT-KTQ I614A Y671A	6.5	93.5	0.2	0.9	5.4
RT-KTQ I614A G672H	2.6	97.4	0.5	1.4	0.8
RT-KTQ I614A R746K	2.1	97.9	0.4	0.9	0.8
RT-KTQ T664K G672A	1.8	98.2	0.2	0.6	0.9
RT-KTQ G668Y Y671A	14.8	85.2	0.1	10.0	4.7
RT-KTQ Y671T R746K	5.8	94.2	0.2	2.6	3.1

Table S3. Rates of dTMP and dAMP incorporation opposite A and m^6A by selected RT-KTQ mutants at constant dNTP concentrations of 100 µM.

DNA polymerase	template	dTMP incorporation rate[a] [min^{-1}]	dAMP incorporation rate[a] [min^{-1}]	rate dTMP/rate dAMP
RT-KTQ	A	50.7 ± 5.6	0.406 ± 0.018	125 ± 15
RT-KTQ	m^6A	22.5 ± 3.0	0.188 ± 0.007	120 ± 17
RT-KTQ I614A	A	169 ± 8	7.17 ± 0.24	22.3 ± 1.4
RT-KTQ I614A	m^6A	154 ± 7	4.79 ± 0.14	32.2 ± 1.7
RT-KTQ G668Y	A	12.3 ± 0.3	0.306 ± 0.004	40.2 ± 1.1
RT-KTQ G668Y	m^6A	1.71 ± 0.07	0.124 ± 0.001	13.8 ± 0.6
RT-KTQ Y671A	A	16.0 ± 0.6	0.259 ± 0.010	61.8 ± 3.3
RT-KTQ Y671A	m^6A	1.72 ± 0.04	0.159 ± 0.006	10.8 ± 0.5
RT-KTQ G672H	A	7.88 ± 0.31	0.0366 ± 0.0008	215 ± 10
RT-KTQ G672H	m^6A	0.646 ± 0.016	0.0163 ± 0.0003	39.6 ± 1.2
RT-KTQ G668Y Y671A	A	2.91 ± 0.11	0.164 ± 0.002	17.7 ± 0.7
RT-KTQ G668Y Y671A	m^6A	0.247 ± 0.003	0.153 ±0.003	1.61 ± 0.04

[a] Data points derive from triplicates. ± describes SD.

Supplementary Figures

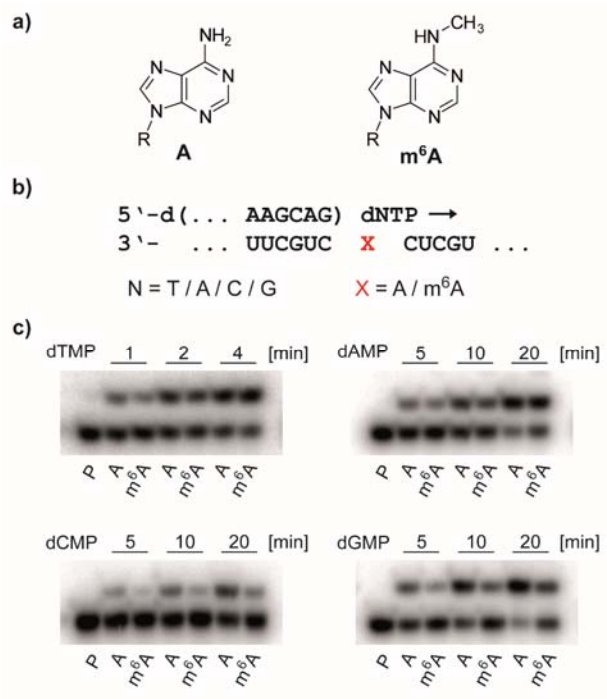

Figure S1. A/m^{6}A discrimination by RT-KTQ for incorporation of each of the four dNTPs. a) Chemical structure of A and m^{6}A. b) Partial primer template sequence. c) Single nucleotide incorporation opposite A and m^{6}A catalyzed by RT-KTQ. 1 nM (dTMP) or 20 nM (dAMP/ dCMP/ dGMP) RT-KTQ and 100 µM of the respective dNTP were applied.

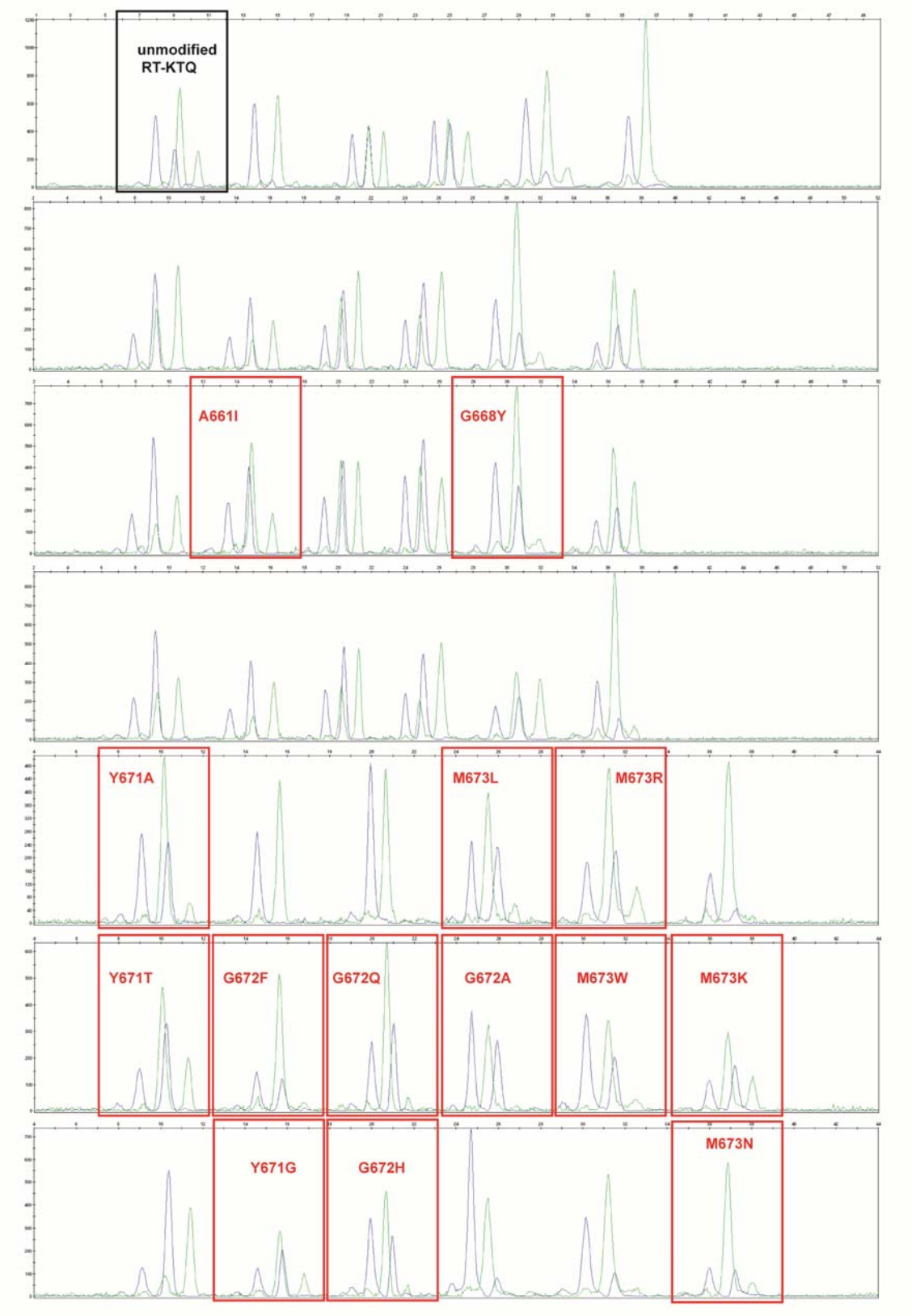

Figure S2. Selected examples of electropherograms to monitor dTMP incorporation by RT-KTQ single mutants. Reactions with template A are shown in blue (FAM-channel), reactions with template m^6A in green (HEX-channel). Differences in retention time for FAM- and HEX-labeled oligonucleotides of the same length derive from differential electrophoretic mobility of the fluorophores. DNA polymerase variants with significant A/m^6A discrimination are highlighted in red. This screening assay was performed for >300 RT-KTQ single mutants. Primer extension was performed in presence of 0.8 µM dTTP.

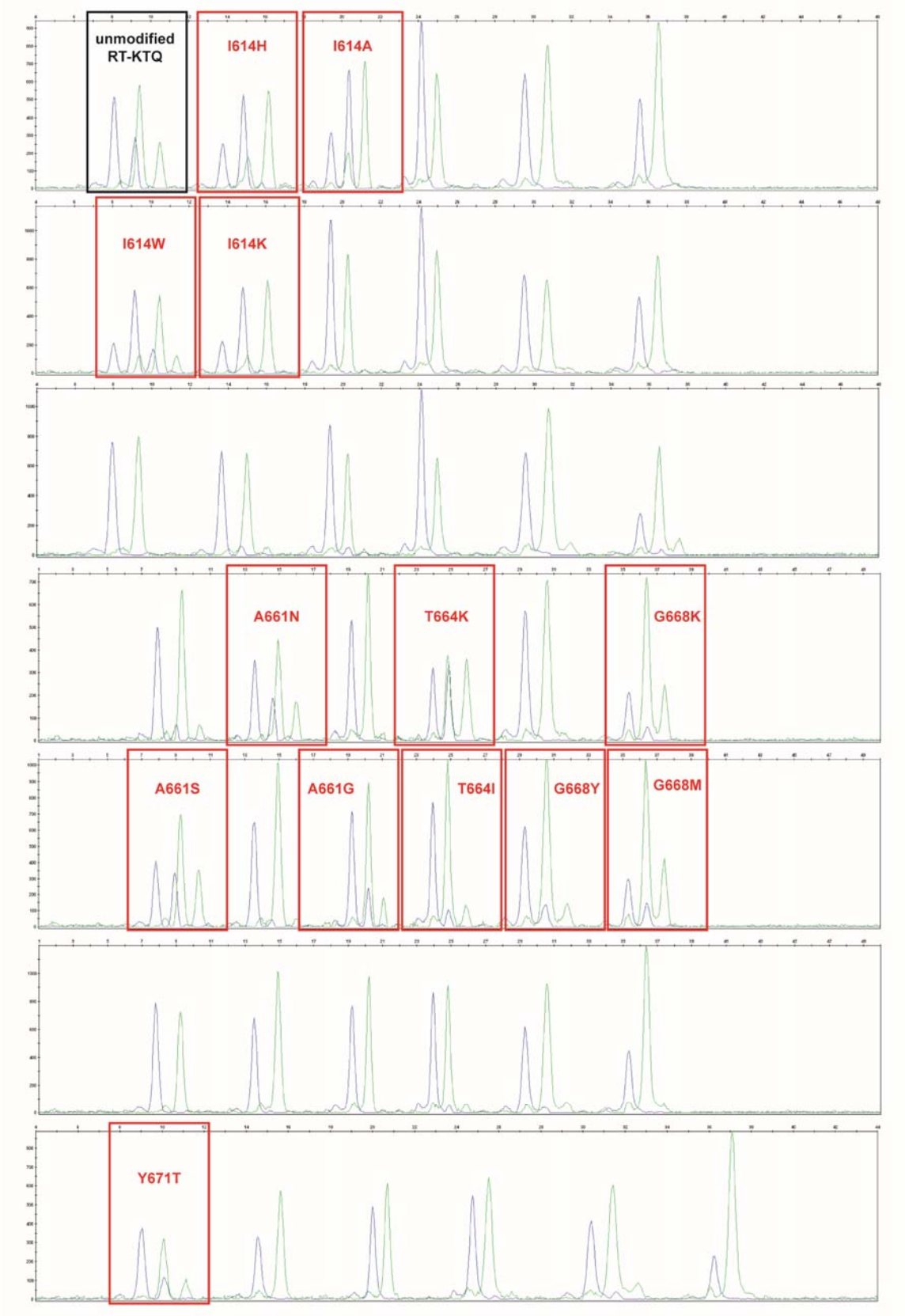

Figure S3. Selected examples of electropherograms to monitor dAMP incorporation by RT-KTQ single mutants. Reactions with template A are shown in blue (FAM-channel), reactions with template m^6A in green (HEX-channel). Differences in retention time for FAM- and HEX-labeled oligonucleotides of the same length derive from differential electrophoretic mobility of the fluorophores. DNA polymerase variants with dAMP discrimination comparable to (or higher than) unmodified RT-KTQ are highlighted in red. This screening assay was performed for >300 RT-KTQ single mutants. Primer extension was performed in presence of 20 µM dATP.

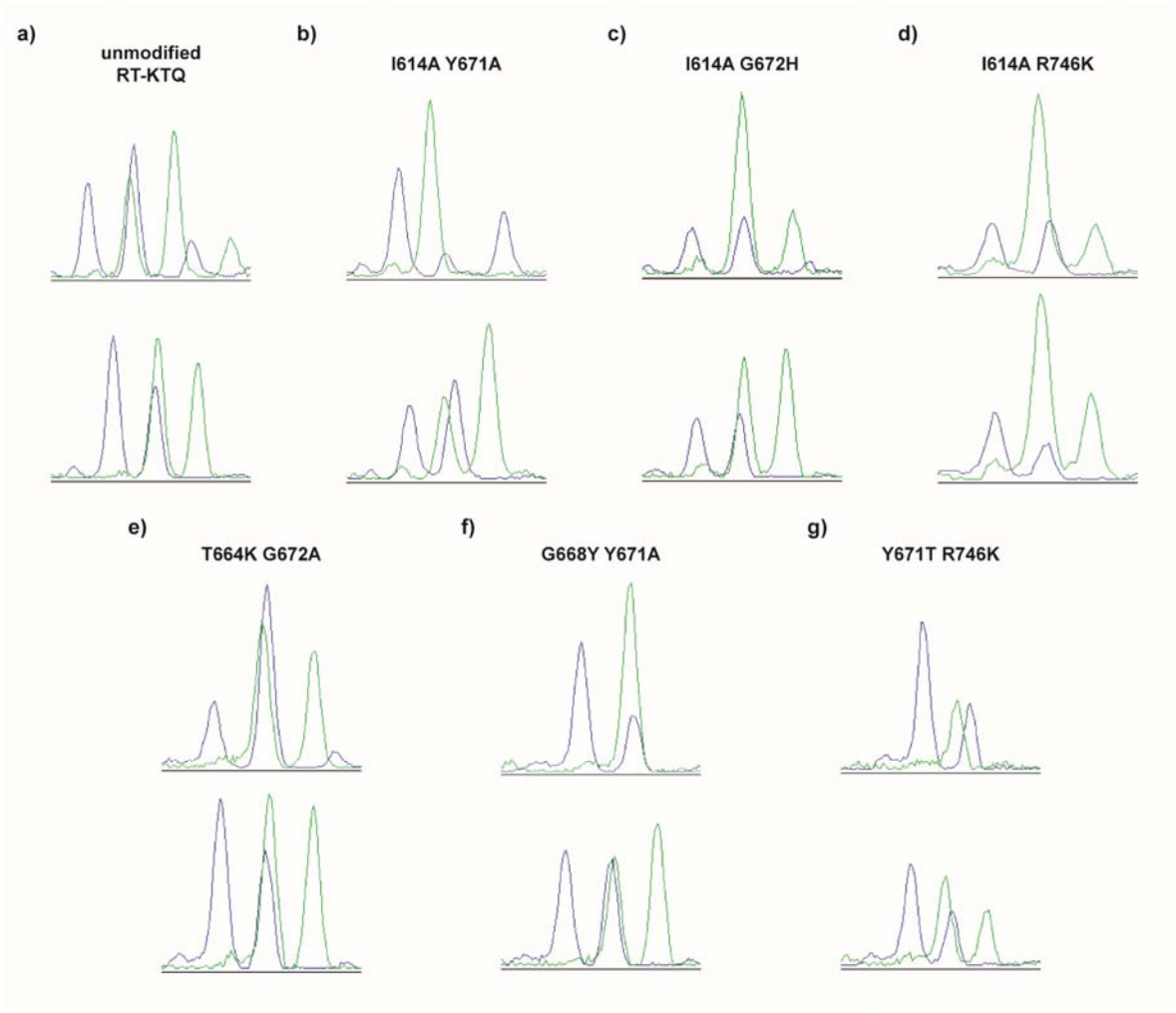

Figure S4. Screening data for dTMP and dAMP incorporation by selected RT-KTQ double mutants. dTMP incorporation (top) and dAMP incorporation (bottom) opposite A (blue / FAM-channel) and m^6A (green / HEX-channel) by unmodified RT-KTQ (a), RT-KTQ I614A Y671A (b), RT-KTQ I614A G672H (c), RT-KTQ I614A R746K (d), RT-KTQ T664K G672A (e), RT-KTQ G668Y Y671A (f) and RT-KTQ Y671T R746K (g). Differences in retention time for FAM- and HEX-labeled oligonucleotides of the same length derive from differential electrophoretic mobility of the fluorophores. For dTMP incorporation 0.8 µM dTTP were applied in primer extension reactions and for dAMP incorporation 20 µM dATP. The screening assay was performed for all possible RT-KTQ double mutants carrying one 'discriminator' mutation (L616T, Y671A, G672H, G672A, G672K, M673T, R746K) and one 'misincorporator' mutation (I614A, A661K, T664K, G668Y, Y671T, F749P). The here depicted mutants delivered the most promising results in the screening and were therefore applied for NGS library preparation.

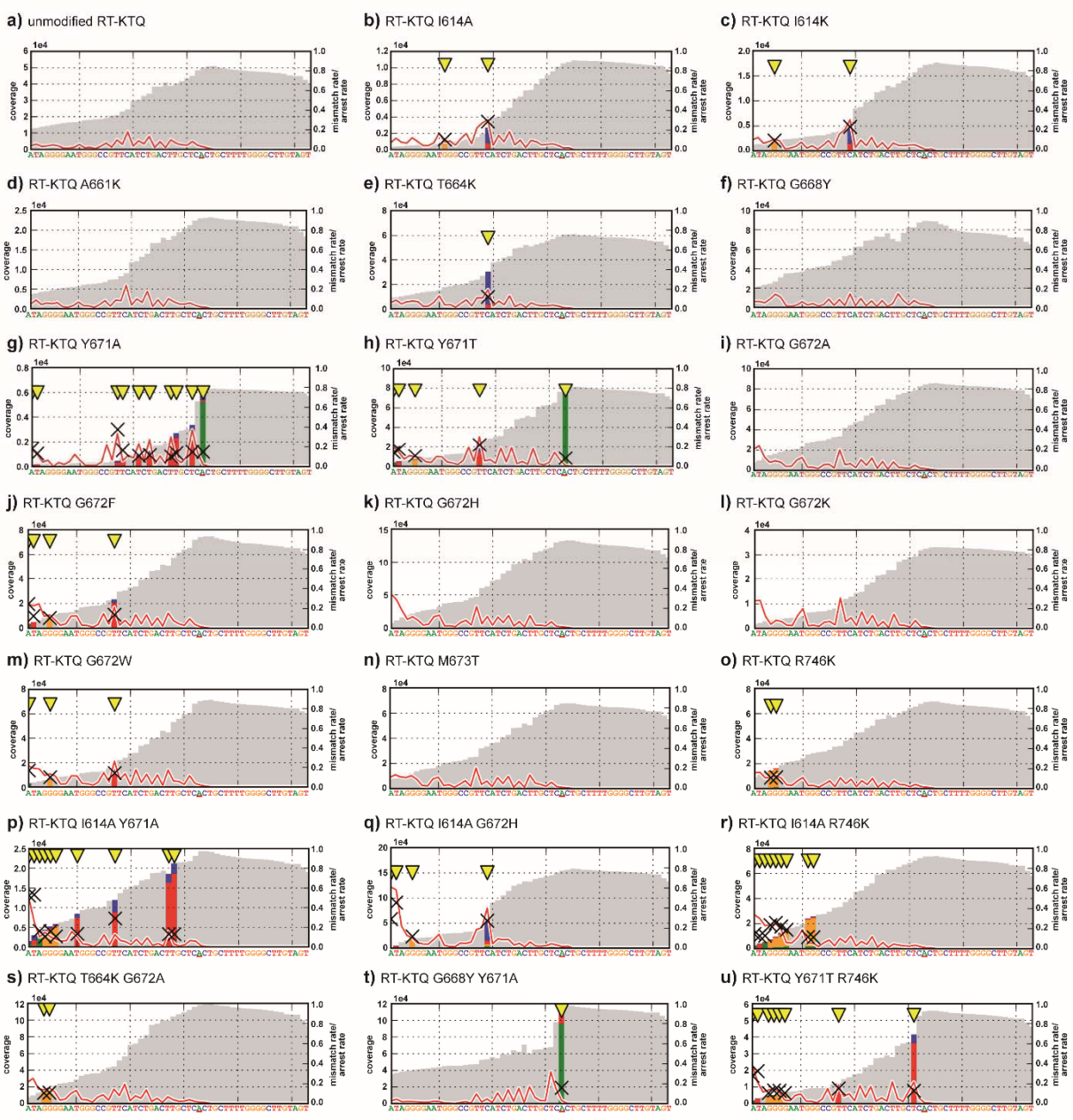

Figure S5. Sequencing profiles of an m^{6}A containing RNA oligonucleotide reverse transcribed by selected RT-KTQ variants. Sequencing profile when employing unmodified RT-KTQ (a), RT-KTQ I614A (b), RT-KTQ I614K (c), RT-KTQ A661K (d), RT-KTQ T664K (e), RT-KTQ G668Y (f), RT-KTQ Y671A (g), RT-KTQ Y671T (h), RT-KTQ G672A (i), RT-KTQ G672F (j), RT-KTQ G672H (k), RT-KTQ G672K (l), RT-KTQ G672W (m), RT-KTQ M673T (n), RT-KTQ R746K (o), RT-KTQ I614A Y671A (p), RT-KTQ I614A G672H (q), RT-KTQ I614A R746K (r), RT-KTQ T664K G672A (s), RT-KTQ G668Y Y671A (t), and RT-KTQ Y671A R746K (u). Sites with error rates >10% are highlighted by 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 by a red underline. Figure was created employing CoverageAnalyzer[8].

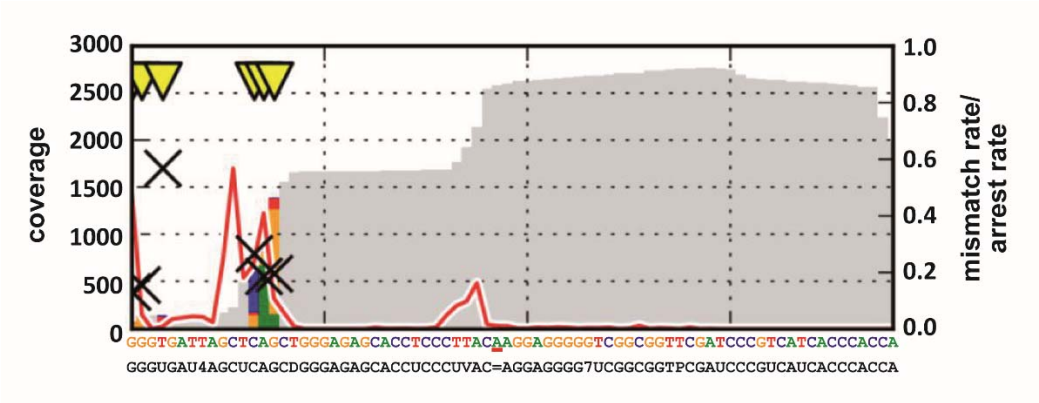

Figure S6. Sequencing profile of *E.coli* tRNA Val reverse transcribed by unmodified RT-KTQ. Sites with error rates > 10% are highlighted by yellow arrows with colored bars indicating the nature of the reads. Mismatch rates are depicted as black crosses, arrest rates as red line. The colored sequence 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; '=' = m6A; '7' = 7-methylguanosine; 'T' = 5-methyluridine; 'P' = pseudouridine).[6] Figure was created employing CoverageAnalyzer[8].

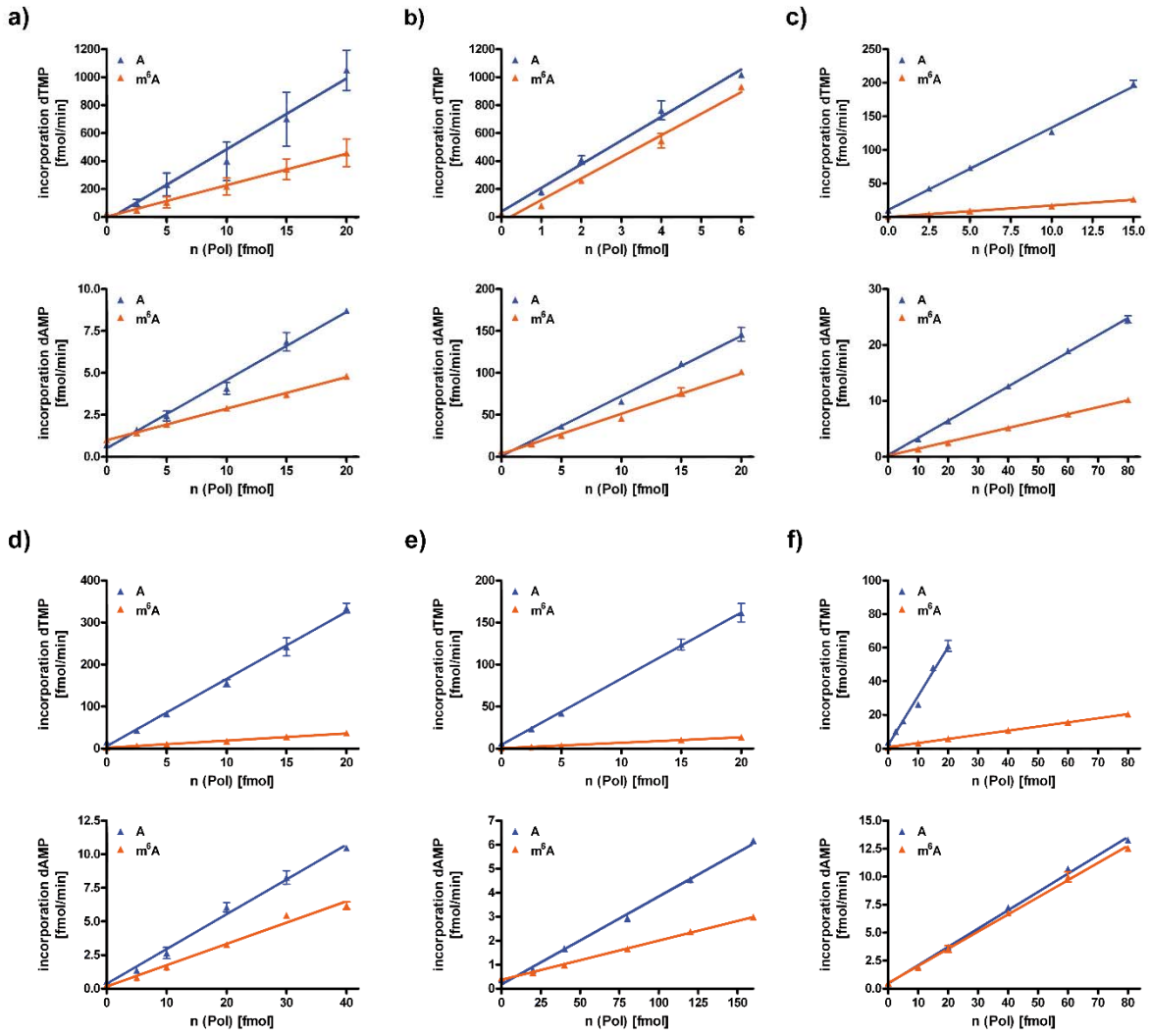

Figure S7. Calculation of dTMP and dAMP incorporation rates opposite A and m^6A. dTMP (top) and dAMP (bottom) incorporation per minute opposite A (blue) and m^6A (orange) catalyzed by RT-KTQ (a), RT-KTQ I614A (b), RT-KTQ G668Y (c), RT-KTQ Y671A (d), RT-KTQ G672H (e) and RT-KTQ G668Y Y671A (f) plotted against the amount of applied enzyme in presence of 100 µM dTTP or dATP. Error bars describe SD (n = 3).

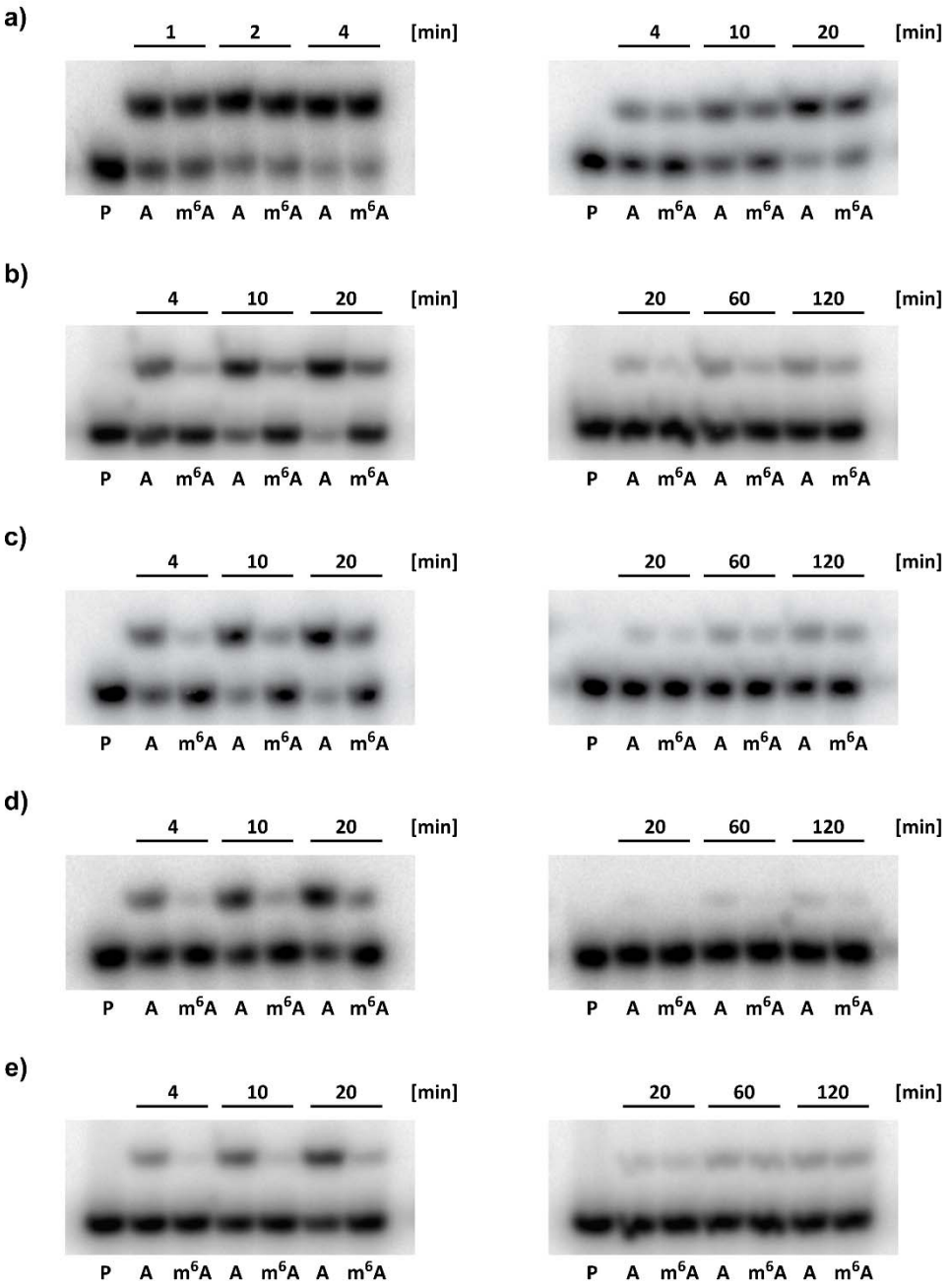

Figure S8. Effects of certain RT-KTQ mutations on m^6A discrimination and dAMP misincorporation. Single nucleotide incorporation of dTMP (left) and dAMP (right) opposite A and m^6A catalyzed by RT-KTQ I614A (a), RT-KTQ G668Y (b), RT-KTQ Y671A (c), RT-KTQ G672H (d) and RT-KTQ G668Y Y671A (e). dTTP/ dATP concentration was 100 μM and enzyme concentration was 1 nM for all reactions. Incubation times were adjusted for each enzyme as indicated.

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