SUPPORTING INFORMATION

Identification of a Selective Polymerase Enables Detection of N^6-methyladenosine in RNA

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MATERIALS AND METHODS

Synthetic RNA oligonucleotides. The phosphoramidite form of N^6-methyladenosine for use in automated RNA synthesis was prepared with modifications over reported literature methods,[1,2] resulting in improved efficiency. The synthetic methods we employed are described below. RNA oligomers were synthesized on an Applied Biosystems 394 synthesizer using standard β-cyanoethyl phosphoramidite chemistry and 2'-*O*-TBDMS-protected ribonucleosides. Phosphoramidites of A, C, G, and U and synthesizer reagents were purchased from Glen Research. Deprotection of the 2'-*O*-TBDMS group and initial purification were carried out using Glen-Pak RNA purification columns according to the instructions provided. RNA oligonucleotides were further purified using polyacrylamide gel electrophoresis. Purity and identity were confirmed with MALDI-TOF mass spectrometry (Stanford Peptide and Nucleic Acid Facility).

Selection of sequence context for kinetics evaluation. *eef2* was identified as a highly-expressed, highly-modified gene using m^6A-specific immunoprecipitation and massively parallel sequencing as described in the literature.[3,4] The region of modification was localized to a 100-nt region in the 3' untranslated region of the gene, given below. A test sequence (bold) was identified based on similarity to the 5-base consensus sequence and minimal secondary structure, as evaluated using DINAmelt.[5]

5'ACAGCAGCCGCCCCAGCCAAGTGGTAGCTGAGACGCGCAAGCGCAAGGGCCTGA

AAGAGGGCATCCCAGCGCTGGACAACTTCCTGGACAAACTGTAGGCAGCCTG**ATAC**

TGCCACATGCTGCACAGTGCCCACCCATCAGAAGACACCTTGAGACTGTCCCACA

GTGCTCCTCTAGAGGCTGCTGGGGCCACCCTGACATCACTCAGCACTCACTTGCTAC

CAATTCTATTTATTTCGGAATTACAAGATAGCGGGAATCTCTCTGCAGGCTGGACTG
GCAGGCTGTGGGGTGGGCGGGACACGGCTCTTAACATTTTCAGAGGGAAACGCGCA
GATGTCCAAAAGTCTAAATAAATGCATTCAGAGGTTTTTGGGGTCCATGGCCAAGTG
GAGTTCCCCCAGAGGGGGAGG-3'

Primers and primer labeling. DNA primers were ordered from the Stanford Peptide and Nucleic Acid Facility and purified by polyacrylamide gel electrophoresis. 5' labeling with ^{32}P was carried out using T4 polynucleotide kinase (Invitrogen) and [γ-^{32}P] ATP (PerkinElmer) according to standard methods and purified by ethanol precipitation.[6]

Primers for kinetic studies were as follows:

5'-GCACTGTGCAGCATGTGGCAG-3'

5'-GCACTGTGCAGCATGTGGCAA-3'

5'-GCACTGTGCAGCATGTGGCAC-3'

5'-GCACTGTGCAGCATGTGGCAT-3'

Primers for rRNA were as follows:

5'-AGCTCGCCTTAGGACACCTGCGT-3' (28s, nt 4189, potential m^6A)

5'-GAGCTCGCCTTAGGACACCTGCG-3' (28s, nt 4190, potential m^6A)

5'-TCCTTCCGCAGGTTCACCTACGGAAACCTTG-3' (18s, nt 1832, m^6A)

5'-GCTGACTTTCAATAGATCGCAGCGAGGGAGC-3' (28s, nt 4984, A)

5'-CCTCTAGATAGTCAAGTTCGACCGTCTTCTCAGCGC-3' (18s, nt 1781, A)

Primers for the 3'-UTR of *bPRL* were as follows:

5'-GGTGACACTATAGAATAGAGATTTTGACATCGCTACAGAG-3' (m^6A)

5'-TTTAAGATGAAACCCATTAGAGCCAAGCATGCAT-3' (A)

Enzyme screening. Commercially-available reverse transcriptases were screened for their ability to incorporate dTTP selectively versus A or m^6A in an RNA template. A 2x stock solution of primer, labeled primer, and template was prepared and annealed in a generic reverse transcription buffer (1x buffer: 50 mM Tris pH 8.3, 75 mM KCl, 5 mM DTT, 3 mM $MgCl_2$). For each reaction, 3 μL of the annealed solution was combined with 2 μL of 2x enzyme. This was heated for 2 min at the reaction temperature, and then 5 μL of a 200 μM TTP solution in 1x buffer was added (for AMV, and *Tth*, the triphosphate buffer was that provided with the enzyme). To terminate the reaction, 10 μL of stop buffer (95% formamide, 25 mM EDTA pH 8.0, 0.01% bromophenol blue, 0.01% xylene cyanol) was added. The reaction times, final concentrations, and temperatures for enzymes were as follows: HIV reverse transcriptase (Worthington Biochemical) – 30s, 0.12 U/μL, 37°C; M-MLV RT (Ambion) – 30s, 0.4 U/μL, 37°C; AMV RT (Invitrogen) – 30s, 0.6 U/μL, 37°C; Maxima RT (Thermo Scientific) – 30s, 0.8 U/μL, 37°C; Superscript II RT (Invitrogen) – 30s, 0.4 U/μL, 37°C; recombinant *Tth* DNA polymerase (Applied Biosciences) – 2 min, 0.005 U/μL, 70°C. The final concentrations were 1 μM for RNA template (5'-AU**X**CUGCCACAUGCUGCACAGUGC-3') and 0.5 μM for primer (5'-GCACTGTGCAGCATGTGGCAG-3').

The reactions were run on a 20% polyacrylamide gel, and exposed overnight using a phosphor screen. A GE Typhoon 9410 gel imager was used in imaging, and ImageQuant 5.2 was used for analysis.

***Tth* polymerase kinetics.** Polymerase kinetics were determined using the standing-start method.[7] As described above, a 2x primer-template solution was annealed and combined with a 2x

solution of enzyme. The mixture was heated for 2 min at 70°C, and then a 2x solution of dTTP (with 10 mM Tris-HCl pH 8.3, 90 mM KCl, and 1 mM $MnCl_2$) was added, and the reaction was allowed to proceed for 1 min at 70°C before being stopped by addition of an equal volume of stop buffer. The dTTP concentration was varied from 5 to 25 μM for sequences containing A and from 20 to 150 μM for sequences containing m^6A. The concentrations of other components were the same as listed above. Reactions were resolved by gel electrophoresis and imaged as described above. Quantitative imaging was carried out using ImageQuant 5.2 software to determine the percent of primer that had been extended. Each band was enclosed in a box of the same area, and the volume calculated. Background subtraction was done manually. When multiple extension products were present, the percent incorporation was calculated by adding the volume of all extension products together and dividing by extension products plus unextended primer. Hanes-Woolf plots were created to obtain kinetics values. Final values were obtained using the average of three or more experimental trials.

Determining m^6A:A ratio. Reactions were carried out as described above, with a 100 μM dTTP concentration and ratios of A- to m^6A- containing oligos in 20% intervals from 0% to 100%. The total RNA concentration was either 1 μM or 0.1 μM. After quantification, the percent incorporation at each concentration was calculated, and the ratio of incorporation was determined by dividing the percent incorporation of a given m^6A-strand concentration by the percent incorporation when 100% A was present.

The points and standard deviations after 5 trials (fraction m^6A (x), relative incorporation (y): 0, 1.0; 0.2, 0.87±0.24; 0.4, 0.70±0.17; 0.6, 0.61±0.11; 0.8, 0.33±0.09; 1.0, 0.22±0.07) were plotted using Excel. Both standard and weighted least squares regression lines were calculated,[8]

using the inverse square of the standard deviation as the weights. Very similar equations were obtained with both methods:

$y = -0.80x + 1.02$ (unweighted)

$y = -0.85x + 1.06$ (weighted to account for relative error in measurements; shown in Figure 2)

We calculated the 95% confidence limits for the relative incorporation when the fraction of m^6A is 0.2 using standard methods[8] and obtained 0.86±0.08, indicating that 20% modification can be detected.

Determining AMV ratio. Samples were prepared side-by-side as described above. For AMV RT, the 2x dTTP solution contained 50 mM tris-acetate pH 7.1, 75 mM potassium acetate, and 8 mM magnesium acetate. The reactions were carried out for one minute at 37°C with 0.06 U/μL of AMV RT. The percent incorporation of a particular site with *Tth* pol was divided by the percent incorporation with AMV RT to determine the ratio.

Isolation of total RNA from 293T cells. 293T cells were cultured in DMEM (Gibco, Life Technologies) supplemented with 1% Pen Strep (Gibco, Life Technologies) and 10% Fetal Bovine Serum (HyClone). Cells where collected with TRIzol (Ambion) and incubated at room temperature for 10 min. After addition of chloroform (J.T.Baker), 20% final volume, cells were centrifuged at maximum speed for 30 minutes and the aqueous phase collected. 1 volume of ethanol was added to the aqueous phase and passed through an RNeasy column (Quiagen). The column was washed once with buffer RPE and the RNA eluted with RNAse free water. The RNA concentration was determined on a NanoDrop 1000 (Thermo Scientific) and RNA was

lyophilized on a FreeZone 4.5 PLUS (Labconco). RNA was re-suspended to 2.2 μg/μl (rRNA detection) or 25 μg/μl.

Detection of m^6A in rRNA. A 2x annealing solution was prepared in a total volume of 15 μL, with 1x reverse transcriptase buffer, 1.5 μL of each radiolabeled primer, 5.0 μL of 2.2 μg/μL total RNA, with unlabeled primer added so that the final (1x) concentration of each would be 0.5 μM. The mixture was heated for 10 min at 95°C, then cooled slowly to room temperature.

3 μL of annealing solution were combined with 2 μL of enzyme and heated at 37°C (AMV RT) or 70°C (*Tth* pol) for two minutes before adding the appropriate 2x dTTP solution (final dTTP concentration: 100 μM) as described above. The reactions were heated for 10 minutes before being terminated by an addition of an equal volume of stop buffer. Reaction products were resolved on a 20% denaturing polyacrylamide gel, which was imaged and quantified as described in the kinetics section. Final enzyme concentrations were 0.05 U/μL for *Tth* Pol and 0.3 U/μL for AMV RT.

Preparation of cells containing 3'-bPRL UTR. The 3'UTR sequence of bovine prolactin was synthetized as a gBlock by IDT and cloned at the ApaI restriction site of pcDNA3-EGFP (Addgene plasmid 13031) by Gibson assembly (New England BioLabs), according to manufacture's protocol. pcDNA3-EGFP or pcDNA3-EGFP plus 3' UTR of bovine prolactin were co-transfected with pDT-CMV-mCherry, kindly supplied by David Thompson, in 293T cells with Lipofectamine 2000, according to manufacturer's protocol. Cells where collected 48h after transfection and RNA extracted as described above.

gBlock sequence (underlined are the sequences for the Gibson assembly, in bold is the putative modified site, and in lowercase is the A site used as a control):

AGTAATCTAGAGGGCCGCCCACATTCCATCCTATCCATTTCTGAGATGGTTCTTAAT

GATCCATTCCCTGGCAAACTTCTCTGAGCTTTATAGCTTTGTaATGCATGCTTGGCTC

TAATGGGTTTCATCTTAAATAAAAACAG**A**CTCTGTAGCGATGTCAAAATCTGGCCCT

ATTCTATAGTG

*Note: We anticipated that the *bPRL* modification site would remain the same when overexpressed in 293T cells as in native cells and that modification levels would be similar. This seems to be the case for expression of the *bPRL* 3'UTR in HeLa[9,10] and CHO[11] cells, but there are no previous reports using 293T cells.

***In vitro* transcription.** As seen in Table 1, *Tth* DNA pol kinetics for incorporation of dTTP opposite A are variable, and this comes into play when designing control primers. Additionally, AMV may have different context dependence than *Tth.*[12] If the result obtained through comparison of control and probe primer incorporation can be attributed to sequence context effects (varies by sequence, but generally if less than 50% difference is seen between the sites), then *in vitro* transcription is needed as an additional control. With an *in vitro*-transcribed control sequence, the same sequence context can be directly compared.

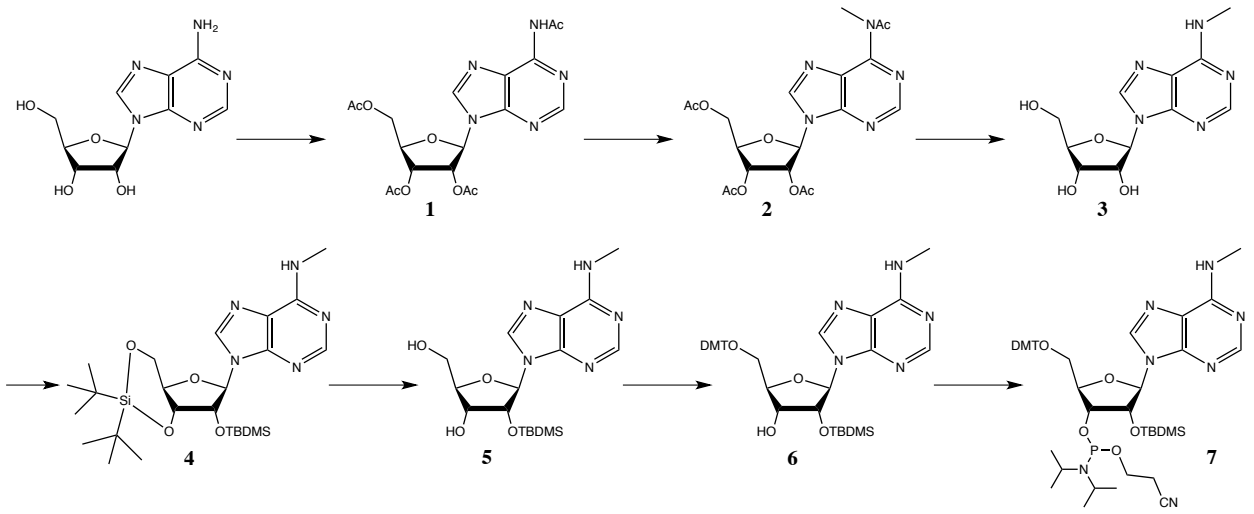
For the *in vitro* transcription reaction, templates with a T7 promoter where generated by PCR with the primers for GFP-bPRL: 5'-TAATACGACTCACTATAGGGCCGCCCACAT TCCATCCTAT-3' and 5'-CTAGAAGGCACAGTCGAGGC-3'. The PCR reaction was purified using the MinElute PCR purification Kit (Quiagen) according to manufacture's protocol. Approximately 1 ug of DNA was used as a template in a transcription reaction using the

MEGAscript T7 Kit (Life Technologies) prepared according to manufacture's protocol. After the IVT reaction, the RNA was purified using the RNAeasy Kit, and the quality of the RNA was confirmed in a 1.5% agarose gel.

Detection of m^6A in mRNA. A 2x annealing solution was prepared in a total volume of 15 μL, with 1x reverse transcriptase buffer, 2.5 μL of each radiolabeled primer, and 1.0 μL of 25 μg/μL total human RNA from 293T cells that had been transformed with the plasmid containing *bPRL*. The mixture was heated for 10 min at 95°C, then cooled slowly to room temperature.

3 μL of annealing solution were combined with 2 μL of enzyme and heated at 37°C (AMV RT) or 55°C (*Tth* polymerase) for two minutes before adding the appropriate 2x dTTP solution (final dTTP concentration: 100 μM) as described above. The reactions were heated for 10 minutes or 1 minute before being terminated by an addition of an equal volume of stop buffer. Reaction products were resolved on a 20% denaturing polyacrylamide gel, which was imaged and quantified as described in the kinetics section. Multiple extension products seen with *Tth* DNA pol in Figure 4 are the result of incorporation of T opposite G (n+2) and A (n+3). The sum of all extension bands was used in quantifying incorporation. Final enzyme concentrations were 0.05 U/μL (10 min) or 0.005 U/μL (1 min) for *Tth* Pol and 0.3 U/μL (10 min) or 0.006 U/μL (1 min) for AMV RT.

SYNTHETIC PREPARATION OF m^6A



N^6-Acetyl-2′,3′,5′-tri-*O*-acetyladenosine (1)

A mixture of adenosine (2 g, 7.48 mmol), pyridine (15 mL), and Ac_2O (7 mL, 74.2 mmol) was stirred at r.t. overnight. The resulting clear solution was heated at 55 °C overnight. The reaction was cooled down and quenched by addition of excess of EtOH. The volatiles were evaporated *in vacuo*. To remove traces of pyridine the residue was co-evaporated successively with portions of EtOH. The resultant foam was dissolved in MeOH (20 mL) and imidazole (0.3 g, 4.4 mmol) was added and the solution was stirred at r.t. overnight. The solution was diluted with $CHCl_3$ (150 mL) and washed with brine (4 × 40 mL). The organic layer was dried (Na_2SO_4) and evaporated to yield **1** (3.3 g, quant.) as a white foam. Characterization matched reported data.[2]

^{1}H NMR ($CDCl_3$, 400MHz): δ = 9.13 (br s, 1H, NH), 8.69 (s, 1H, H-8), 8.23 (s, 1H, H-2), 6.22 (d, J = 5.4 Hz, 1H, H-1′), 5.95 (dd, $J_1 = J_2$ = 5.7 Hz, 1H, H-2′), 5.66 (dd, J_1 = 5.6 Hz, J_2 = 4.4 Hz, 1H, H-3′), 4.41 – 4.48 (m, 2H, H-5′a + H-4′), 4.37 (dd, J_1 = 12.9 Hz, J_2 = 5.4 Hz, 1H, H-5′b), 2.63 (s, 3H, can), 2.15 (s, 3H, AcO), 2.10 (s, 3H, AcO), 2.07 (s, 3 H, AcO).

^{13}C NMR ($CDCl_3$, 100 MHz): δ 179.6, 169.8, 169.6, 152.9, 151.3, 142.7, 122.5, 86.7, 80.6, 77.3, 70.8, 63.2, 26.0, 21.0, 20.8, 20.6.

HRMS [+ scan]: calculated m/z for $C_{18}H_{22}N_5O_8$ 436.1463; observed 436.1459.

N^6-Acetyl-2′,3′,5′-tri-*O*-acetyl-N^6-methyladenosine (2)

To a stirred solution of **1** (3.1 g, 7.1 mmol) in anhydrous MeCN under Ar-atmosphere, DBU (3.2 mL, 21.4 mmol) and MeI (1.1 mL, 17.8 mmol) were added at r.t.. The resulting mixture was stirred at r.t. for 8h. The mixture was diluted with 150 mL CH_2Cl_2 and extracted with 50 mL 0.5 M HCl and three times with 100 mL brine. The organic layer was dried (Na_2SO_4) and the solvents were removed to yield a pale yellow foam. The product appeared to be 95 % pure by TLC and was used for the next step without further purification. An analytical sample was purified by column chromatography (EtOAc/acetone 5:1). Characterization matched reported data.[2]

^{1}H NMR ($CDCl_3$, 400MHz): δ = 8.76 (s, 1H, H-8), 8.76 (s, 1H, H-8), 8.19 (s, 1H, H-2), 6.23 (d, J = 5.1 Hz, 1H, H-1′), 5.95 (dd, $J_1 = J_2$ = 5.7 Hz, 1H, H-2′), 5.67 (dd, J_1 = 5.6 Hz, J_2 = 4.7 Hz, 1H, H-3′), 4.42 – 4.51 (m, 2H, H-5′a + H-4′), 4.34 – 4.42 (m, 1H, H-5′b), 3.62 (s, 3H, NCH_3), 2.34 (s, 3H, can), 2.15 (s, 3H, AcO), 2.12 (s, 3H, AcO), 2.09 (s, 3H, AcO).

^{13}C NMR ($CDCl_3$, 100 MHz): δ 172.0, 170.5, 169.8, 169.6, 154.5, 152.8, 152.3, 142.1, 127.1, 87.0, 80.6, 73.3, 70.7, 63.2, 35.5, 24.5, 21.0, 20.8, 20.7.

HRMS [+ scan]: calculated m/z for $C_{19}H_{24}N_5O_8$ 450.1619; observed 450.1626.

N^6-Methyladenosine (3)

Compound **2** (3.0 g, 6.68 mmol) was dissolved in 25 mL 8 M $MeNH_2$ in EtOH under Ar-atmosphere. The solution was stirred for 3 h at r.t.. The solvents were removed and the residue was dissolved in 150 mL EtOAc and 15 ml EtOH. The organic layer was extracted with 150 mL brine and the aqueous layer was washed ten times with 100 mL portions of EtOAc. The organic layers were dried (Na_2SO_4) and the solvents were removed to yield a yellow solid. 100 mL

EtOAc and 100 mL Et_2O were added to the residue and the mixture was cooled to 4°C overnight. The white precipitate was collected by filtration and washed with Et_2O to yield **3** as a white powder (1.3 g, 4.62 mmol, 69 %). Characterization matched reported data.[2]

^{1}H NMR (400 MHz, Methanol-d_4) δ 8.23 (s, 1H, H-8), 8.22 (br s, 1H, H-2), 5.94 (d, J = 6.5 Hz, 1H, H-1′), 4.74 (dd, J = 6.5, 5.1 Hz, 1H, H-2′), 4.32 (dd, J = 5.1, 2.5 Hz, 1H, H-3′), 4.17 (d, J = 2.5 Hz, 1H, H-4′), 3.88 (dd, J = 12.5, 2.4 Hz, 1H, H-5′a), 3.74 (dd, J = 12.6, 2.6 Hz, 1H, H-5′b), 3.09 (s, 3H, NCH_3).

HRMS [+ scan]: calculated m/z for $C_{11}H_{16}N_5O_4$ 282.1197; observed 282.1193.

2'-*O*-(*tert*-butyldimethylsilyl)-3',5'-*O*-(di-*tert*-butylsilylene)-N^6-methyladenosine (4)

To a stirred solution of **3** (1.05 g, 3.7 mmol) in 20 mL anhydrous DMF at 0°C was added dropwise di-*tert*-butylsilyl bis(trifluoromethanesulfonate) (1.44 mL, 4.1 mmol). The resulting mixture was stirred for 30 min at 0°C, then 15 min at r.t.. Imidazole (1.01 g, 14.8 mmol) was added, followed by *tert*-butyldimethylsilyl chloride (1.12 g, 7.4 mmol). After stirring 1 h at r.t., the reaction mixture was heated to 60°C for 3 h. The mixture was evaporated to a syrup, then dissolved in Et_2O and extracted with water. The organic layer was dried over Na_2SO_4, filtered and evaporated *in vacuo*. Crude product was purified by silica column chromatography (Hexanes/EtOAc 3:1) to give **4** (1.4 g, 71 %) as a white foam.

^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.35 (s, 1H, H-8), 7.75 (s, 1H, H-2), 6.21 (m, 1H, H-1′), 4.60 (d, J = 4.6 Hz, 1H, H-2′), 4.53 (dd, J = 9.4, 4.7 Hz, 1H, H-3′), 4.46 (dd, J = 9.1, 5.0 Hz, 1H, H-5′b), 4.25 – 4.15 (m, 1H, H-4′), 4.01 (dd, J = 10.6, 9.2 Hz, 1H, H-5′a), 3.17 (br s, 3H, NCH_3), 1.11 – 1.06 (s, 9H, tBu), 1.03 (s, 9H, tBu), 0.91 (s, 9H, tBu), 0.12 (s, 3H, $SiCH_3$), 0.12 (s, 3H, $SiCH_3$).

HRMS [+ scan]: calculated m/z for $C_{25}H_{46}N_5O_4Si_2$ 536.3083; observed 536.3094.

2'-*O*-(*tert*-butyldimethylsilyl)-N^6-methyladenosine (5)

A stirred solution of **4** (1.2 g, 2.2 mmol) in 30 mL anhydrous THF was cooled to 0°C. Diluted HF-pyridine (1.2 mL HF-pyridine + 1.2 mL pyridine) was added slowly at 0°C. The mixture was allowed to warm to r.t. and stirred for 30 min. 2 mL pyridine and 60 mL CH_2Cl_2 were added to quench the reaction. The mixture was extracted with sat. aq. $NaHCO_3$ and brine. The organic layers were dried (Na_2SO_4) and the solvents were removed *in vacuo*. The crude product was purified by column chromatography (CH_2Cl_2/EtOAc 1:1) to yield **5** (650 mg, 1.6 mmol, 73 %) as a white foam. Characterization matched reported data.[1]

^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.37 (s, 1H, H-8), 7.72 (s, 1H, H-2), 6.79 (d, *J* = 12.1 Hz, 1H, 5′-OH) 6.00 (br s, 1H, N-H), 5.73 (d, *J* = 7.4 Hz, 1H, H-1′), 5.14 (dd, *J* = 7.4, 4.8 Hz, 1H, H-2′), 4.42 – 4.29 (m, 2H, H-3′ + H-4′), 3.95 (dd, *J* = 12.9, 1.7 Hz, 1H, H-5′a), 3.74 (t, *J* = 12.2 Hz, 1H, H-5′b), 3.19 (s, 3H, NCH_3), 2.85 (s, 1H, 3′-OH), 0.79 (s, 9H, tBu), -0.18 (s, 3H, $SiCH_3$), -0.39 (s, 3H, $SiCH_3$).

HRMS [+ scan]: calculated m/z for $C_{17}H_{30}N_5O_4Si$ 396.2062; observed 396.2068.

2'-*O*-(*tert*-Butyldimethylsilyl)-5'-*O*-(4,4'-dimethoxytrityl)-N^6-methyladenosine (6)

To a stirred solution of **5** (540 mg, 1.4 mmol) in anhydrous pyridine (15 mL) was added 4,4′-Dimethoxytrityl chloride (570 mg, 1.7 mmol) and DMAP (33 mg, 0.27 mmol) and the resulting mixture was stirred at r.t. overnight under Argon atmosphere. The solvent was removed *in vacuo* and the residue was purified by column chromatography (CH_2Cl_2 to CH_2Cl_2/EtOAc 4:1 + 1% Net_3). **6** was obtained as a white foam (740 mg, 1.1 mmol, 76%). Characterization matched reported data.[1]

^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.32 (br s, 1H, H-8), 7.96 (s, 1H, H-2), 7.51 – 7.40 (m, 2H, DMTr), 7.40 – 7.14 (m, 7H, DMTr), 6.80 (dd, *J* = 8.6, 1.4 Hz, 4H, DMTr), 6.02 (m, 2H, N-H + H-1´), 5.00 (dd, *J* = 5.9, 4.7 Hz, 1H, H-2´), 4.33 (m, 1H, H-3´), 4.25 (m, 1H, H-4´), 3.77 (s, 6H, OCH_3), 3.52 (dd, *J* = 10.6, 3.2 Hz, 1H, H-5´a), 3.37 (dd, *J* = 10.5, 3.8 Hz, 1H, H-5´b), 3.16 (s, 3H, NCH_3), 2.80 (br s, 1H, 3´OH), 0.83 (s, 9H, tBu), -0.02 (s, 3H, $SiCH_3$), -0.14 (s, 3H, $SiCH_3$).

HRMS [+ scan]: calculated m/z for $C_{38}H_{48}N_5O_6Si$ 698.3368; observed 698.3376.

2'-*O*-(*tert*-Butyldimethylsilyl)-3'-*O*-(2-cyanoethyl-*N*,*N*-diisopropylphosphino)-5'-*O*-(4,4'-dimethoxytrityl)-N^{6}-methyladenosine (7)

To a stirred solution of **6** (100 mg, 0.14 mmol) in anhydrous CH_2Cl_2 (5 mL) was added N,N-Diisopropylethylamine (0.10 mL, 0.56 mmol) and 2-cyanoethyl-N,N-(diisopropylamino)-chlorophosphoramidite (100 mg, 0.42 mmol) and the resulting mixture was stirred at r.t. 3h under Argon atmosphere. The solution was directly applied to column chromatography (CH_2Cl_2/actetone 8:1 + 1% Et_3N). **7** was obtained as a white foam (110 mg, 0.12 mmol, 85%). Characterization matched reported data.[1]

^{31}P NMR (400 MHz, Chloroform-*d*) δ 151.90 and 149.96 ppm.

HRMS [+ scan]: calculated m/z for $C_{47}H_{65}N_7O_7SiP$ 898.4447; observed 898.4467.

Table S1. MALDI-MS characterization data for synthetic RNAs containing m^6A and A in varied contexts.

RNA sequences (5'→3')	X=A		X=m^6A	
	predicted mass (Da)	experimental mass (Da)	predicted mass (Da)	experimental mass (Da)
AU**X** CUG CCA CAU GCU GCA CAG UGC	7611.64	7612.753	7625.67	7623.948
AG**X** CUG CCA CAU GCU GCA CAG UGC	7650.68	7651.573	7664.71	7661.423
AG**X** UUG CCA CAU GCU GCA CAG UGC	7651.67	7651.600	7665.70	7664.157
AC**X** UUG CCA CAU GCU GCA CAG UGC	7611.64	7610.698	7625.67	7623.481
AC**X** GUG CCA CAU GCU GCA CAG UGC	7650.68	7650.880	7664.71	7661.366
AA**X** GUG CCA CAU GCU GCA CAG UGC	7674.71	7672.941	7688.74	7687.370
AA**X** AUG CCA CAU GCU GCA CAG UGC	7658.71	7658.565	7672.74	7671.340
AU**X** AUG CCA CAU GCU GCA CAG UGC	7635.67	7632.884	7649.70	7647.016
GG**X** CUG CCA CAU GCU GCA CAG UGC	7664.67	7664.237	7678.70	7679.802
GA**X** CUG CCA CAU GCU GCA CAG UGC	7650.68	7649.469	7664.71	7665.348

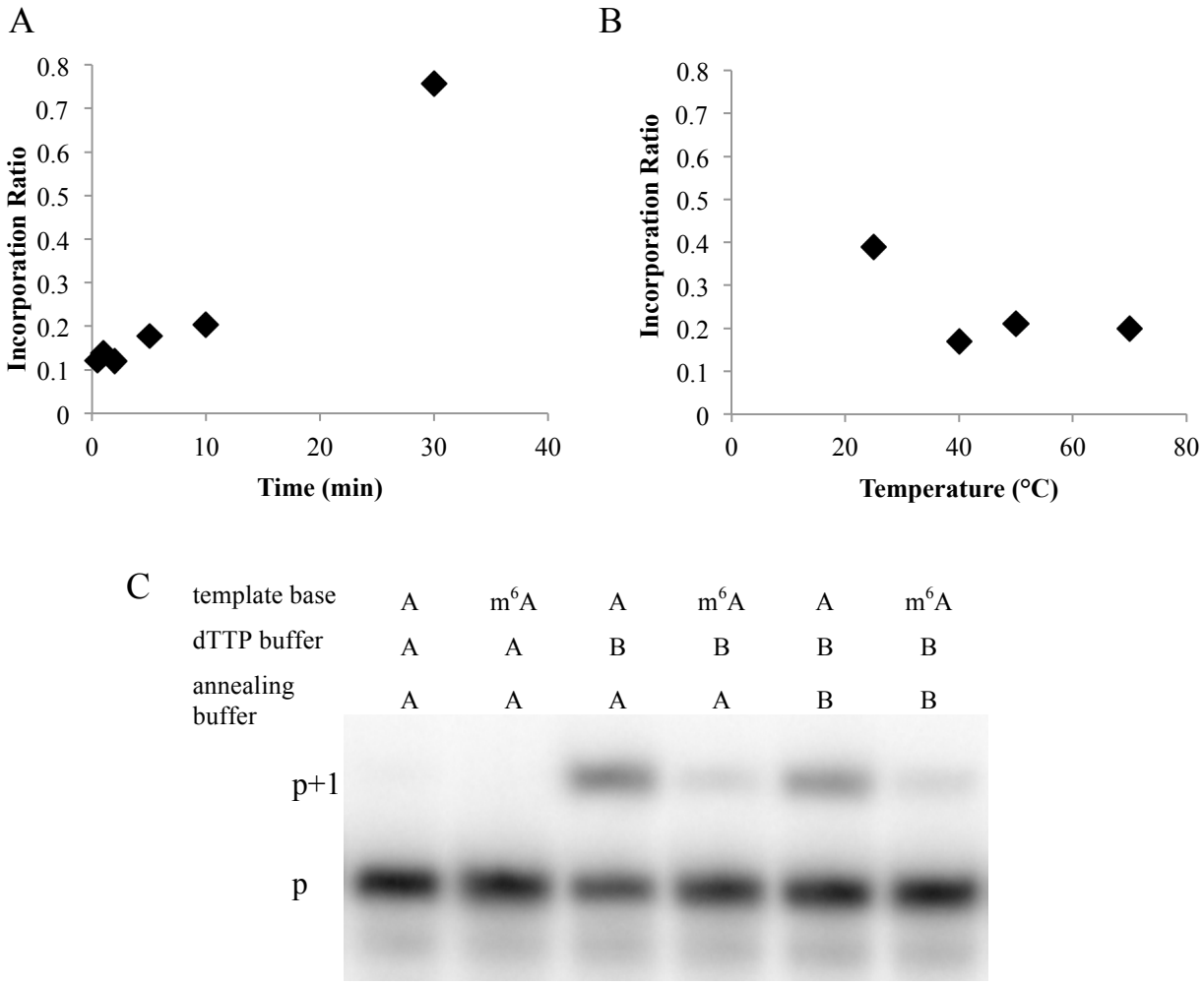


Figure S1. Conditions screened for determination of m^6A:A ratio. Experiments were performed with 1 μM RNA, 0.5 μM DNA primer, and 100 μM dTTP, measuring the single nucleotide incorporation of T vs. A or m^6A. (A) Increasing reaction time decreased the ability of *Tth* DNA pol to distinguish between m^6A and A; this effect was more pronounced at lower RNA concentrations. (B) Temperatures above 40°C had little impact on selectivity; lower temperatures lowered measured selectivity. (C) Mn^{2+} is required for the reaction, but the presence of Mg^{2+} as well has no measurable effect. Buffer A: 50 mM Tris pH 8.3, 75 mM KCl, 5 mM DTT, 3 mM $MgCl_{2;}$; Buffer B: 10 mM Tris-HCl pH 8.3, 90 mM KCl, and 1 mM $MnCl_2$.

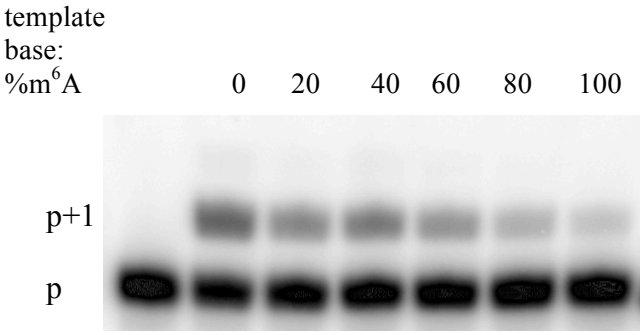


Figure S2. Incorporation is dependent on percent m^6A. Representative autoradiogram shows decreasing incorporation at constant RNA concentration with an increasing percentage of m^6A at the target site. Conditions: 1 μM RNA, 0.5 μM DNA primer, 100 μM dTTP, 0.005 U/μL *Tth* DNA pol; 1 min reaction at 70°C with a final buffer composition of 30 mM Tris pH 8.3, 82.5 mM KCl, 2.5 mM DTT, 1.5 mM $MgCl_2$, and 0.5 mM $MnCl_2$.

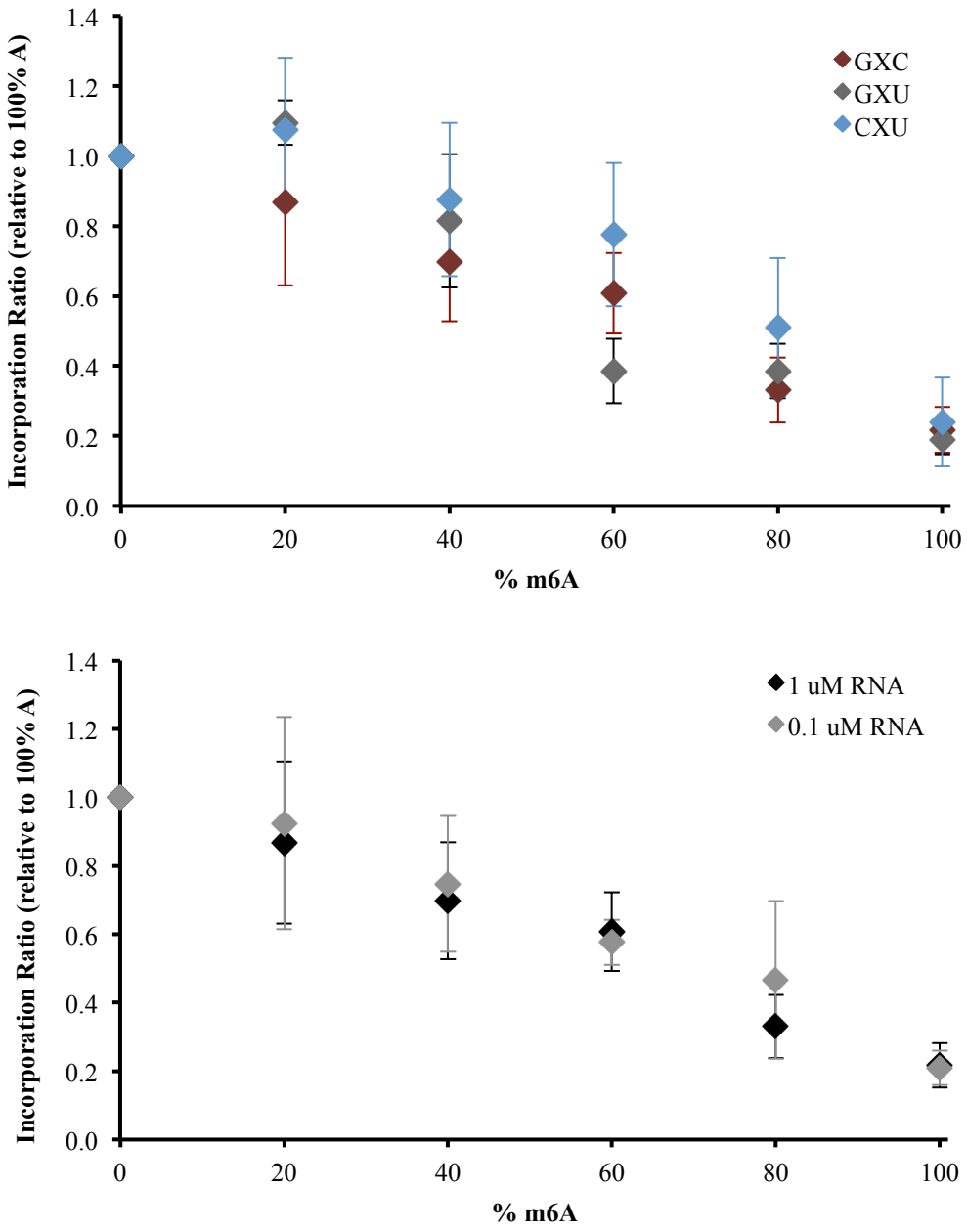


Figure S3. Correlation between *Tth*-mediated dTTP incorporation and percent m6A is independent of context and RNA concentration. (A) Different sequence contexts show similar correlation of incorporation relative to % m^6A in the sample. See Table S1 for full template sequences. Error bars: standard deviation from 2 trials. (B) The correlation between % m^6A and relative primer extension is maintained at a lower (0.1 μM) RNA concentration. Error bars: standard deviation from 3 trials.

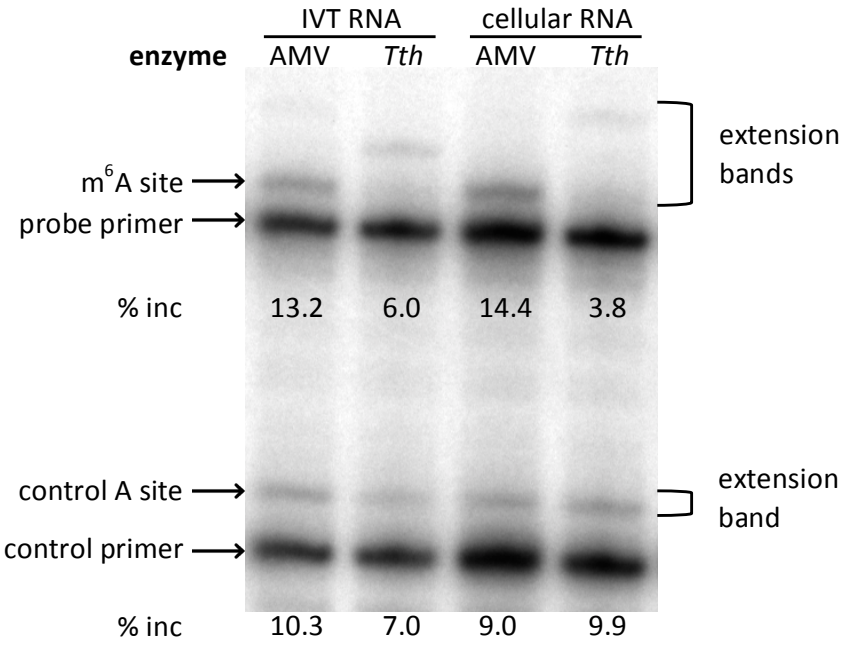
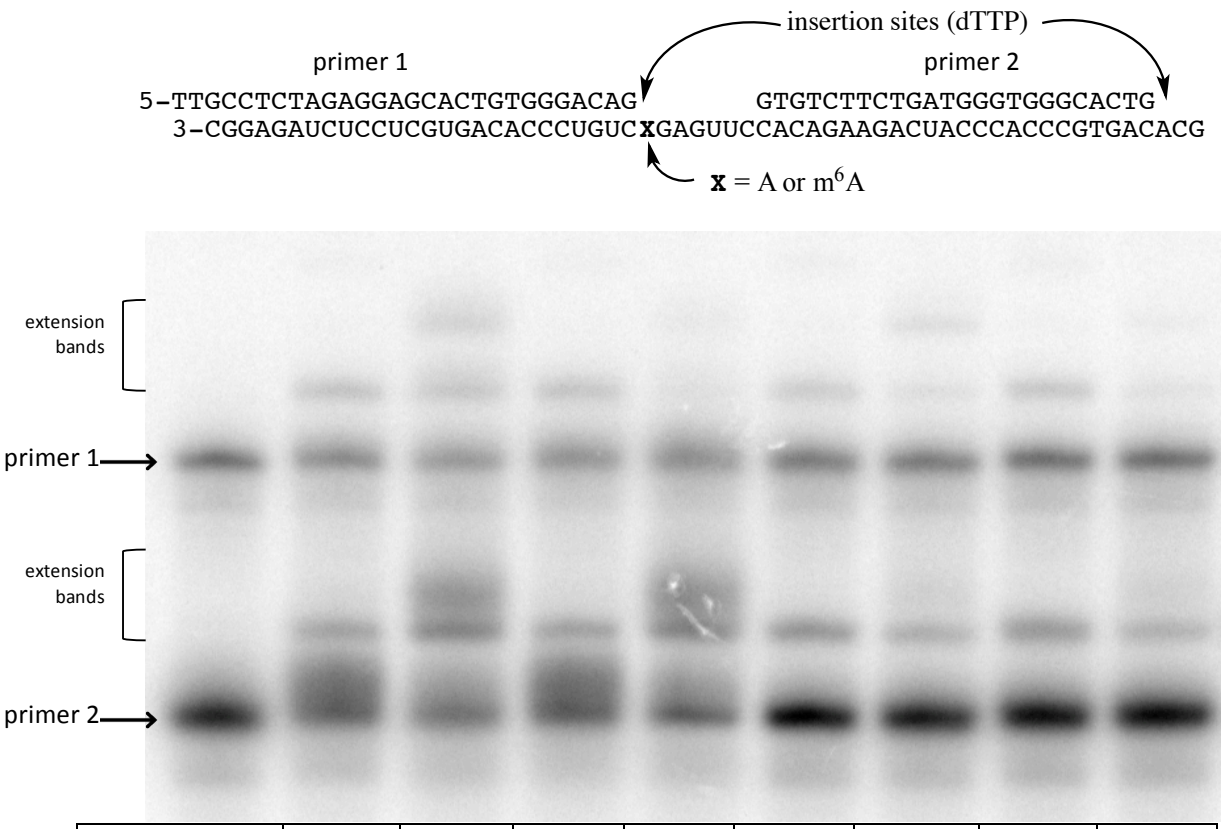


Figure S4. Figure 4 from text showing quantification of the bands as percent incorporation at each site.



m^6A	-	-	+	+	-	-	+	+
enzyme	AMV	*Tth*	AMV	*Tth*	AMV	*Tth*	AMV	*Tth*
RNA concentration	1 µM	1 µM	1 µM	1 µM	0.1 µM	0.1 µM	0.1 µM	0.1 µM
AMV ratio (primer 1)	1.9		0.5		0.8		0.4	
AMV ratio (primer 2)	3.2		3.0		0.8		0.8	
X:A ratio		0.68		0.22		0.81		0.42

Figure S5. Validation of controls for *Tth* pol incorporation of dTTP at m^6A and A-containing sites. The ratios of T incorporation between the two sites and for *Tth* DNA Pol relative to AMV RT were calculated. Primer 2 shows greater incorporation than primer 1 even when A is present at both sites. While in some cases (0.1 µM) AMV RT can be used to normalize the difference in *Tth* pol incorporation between the two sites, this is not true at all sequence contexts and concentrations.

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