Supporting Information

Table of Contents for Supporting Information

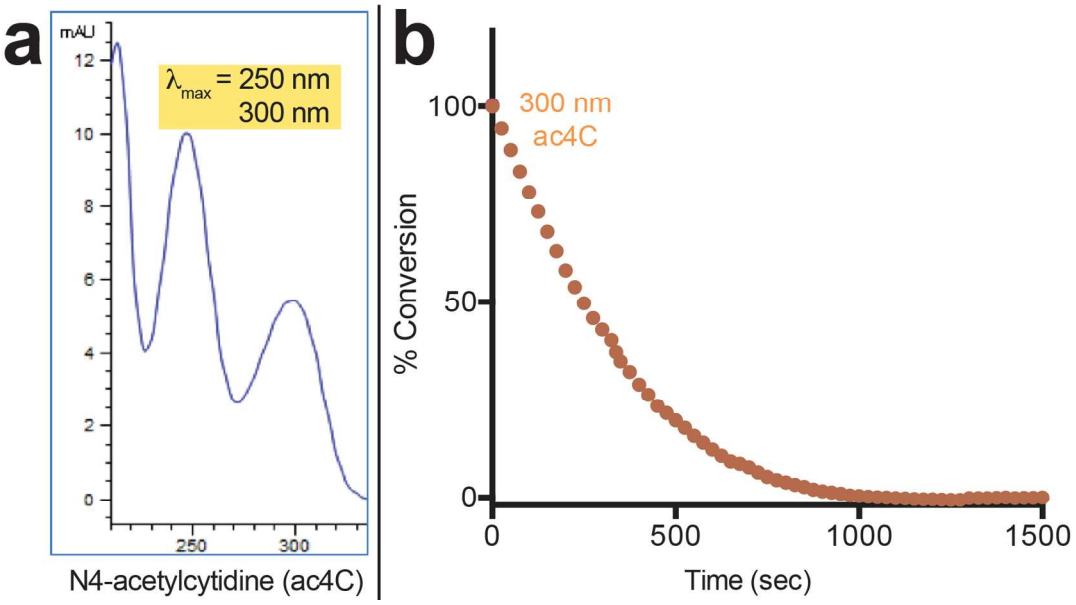


Figure S1. Stability of ac4C to reductive amination conditions. (a) Absorbance spectrum of ac4C. (b) In model reactions, treatment of ac4C with $NaBH_4$ causes rapid loss of its characteristic absorbance at 300 nm. Reaction conditions: (0.25 mM ac4C [free nucleoside], 100 mM $NaBH_4$, ddH_2O).

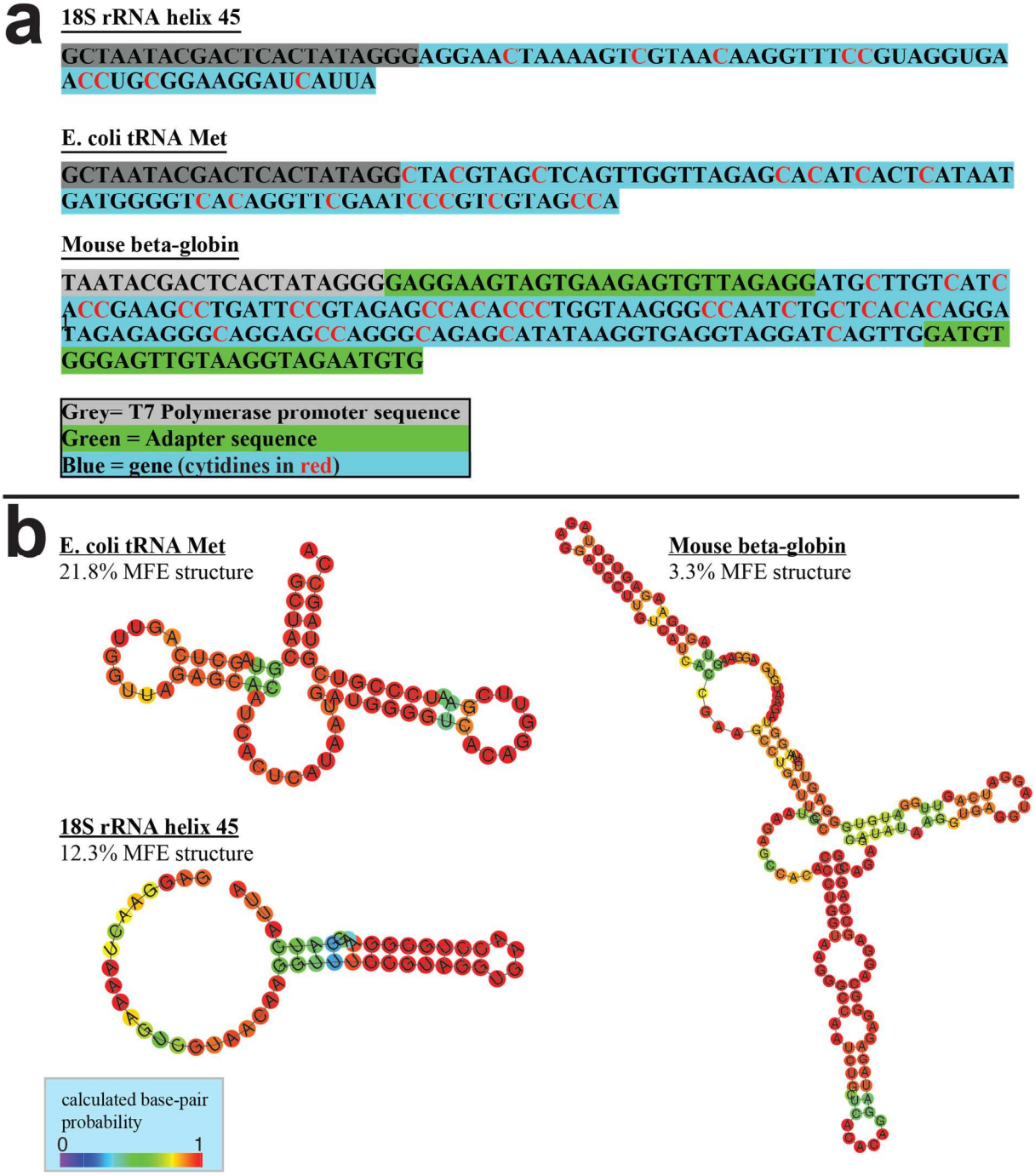


Figure S2. (a) Templates used for in vitro transcription reactions. Cytidines produced in the cognate RNA transcripts are highlighted in red. (b) In silico analysis of structure of RNA probes. Residues are colored according to their calculated base-pair probability. "% MFE structure" refers to the calculated frequency of the minimum free energy (MFE) structure in the structural ensemble, with a higher percentage corresponding to a structure predicted as more ordered. Predictions were performed using the RNAFold webserver (http://rna.tbi.univie.ac.at//cgi-bin/RNAWebSuite/RNAfold.cgi)

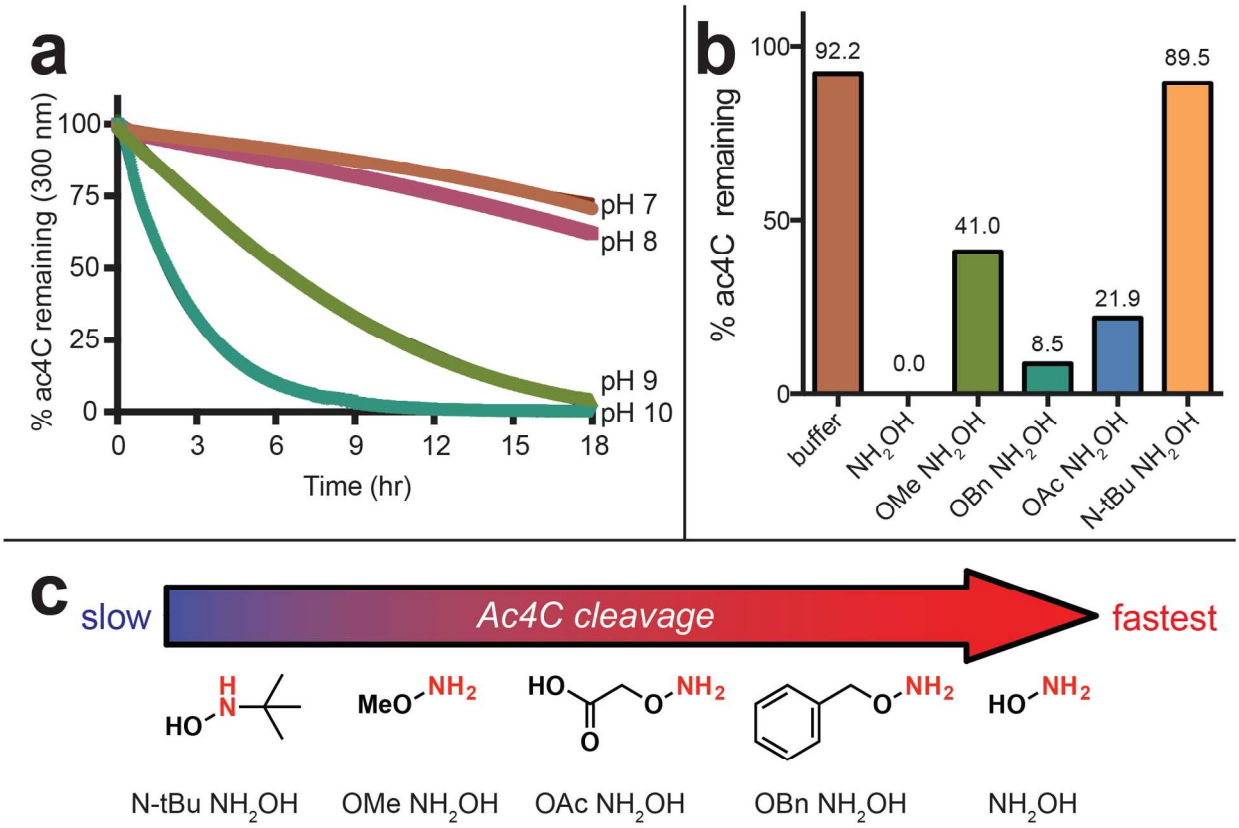


Figure S3. Chemical deacetylation of ac4C. (a) Hydrolysis of ac4C (free nucleoside) is pH-dependent. Reaction conditions: (0.25 mM ac4C [free nucleoside], buffer [Tris pH 7.0/8.0; $NaCO_3$ pH 9.0/10.0], 37 °C). (b) Percent ac4C (free nucleoside) remaining after incubation with a variety of nucleophiles for 5 hrs. Reaction conditions: (0.25 mM ac4C [free nucleoside], 50 mM nucleophile, Tris pH 7.0). (c) Summary of relative reactivity of nucleophiles towards ac4C.

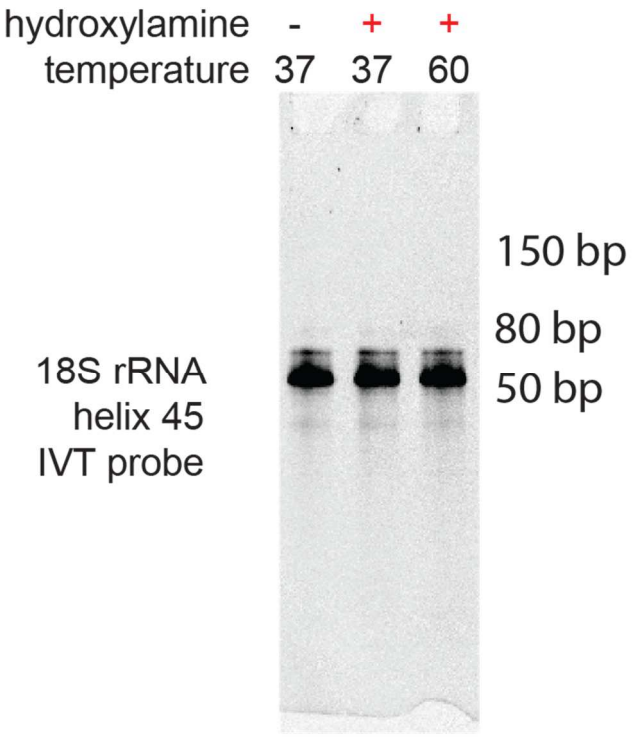


Figure S4. Conditions for mild cleavage of ac4C (50 mM hydroxylamine, pH 8.0, 1 h) do not cause detectable cleavage of the RNA backbone. Full gel image with molecular weight ladder can be found in Supporting Information (below).

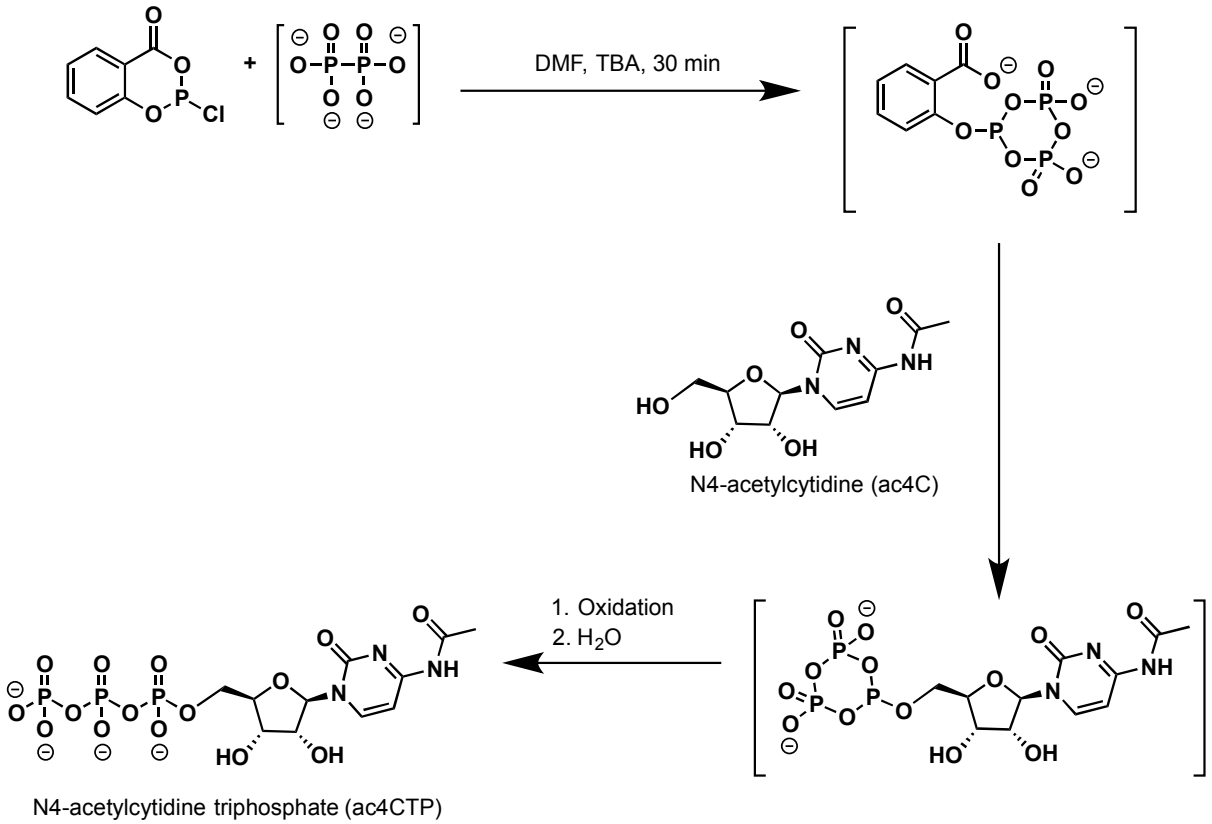
Supplementary materials and methods

Unless otherwise specified, all chemicals and solvents were purchased from Sigma, VWR, or Fisher and used without further purification. Reaction solvents were purchased from Fisher and Aldrich and purified by passage through an aluminum oxide column. N-acetylcytidine (AK112189) was purchased from Ark Pharm and purified by silica gel chromatography prior to use. Sodium periodate, ethylene glycol, potassium carbonate, sodium borohydride, sodium cyanoborohydride were purchased from Sigma. Tributylammonium pyrophosphate (P8533), 2-chloro-1,3,2,-benzodioxaphosphorin-4-one (32412) and tributylamine (9078) were purchased from Sigma and used immediately upon receipt in the phosphitylation reaction. Bovine serum albumin (PI77110) and mariculture keyhole limpet hemocyanin (PI77653) were obtained from Fisher. Ovalbumin was obtained from Aldrich (A5503). Templates for in vitro transcription reactions and primers for amplification were purchased from Integrated DNA Technologies (Coralville, IA). In vitro transcription reactions were analyzed by agarose gel electrophoresis and visualized using ethidium bromide. HPLC purification was performed using an Agilent 1250 Infinity HPLC equipped with a semi-preparative Phenomenex Gemini C18 column (150 x 21.2 mm, 10 μm). LC-MS analyses of ac4CTP were performed on a Shimadzu 2020 LC-MS system. LC-MS/MS analyses of ac4C were performed using a reverse phase chromatography (Shimadzu LC-20AD) coupled to a triple-quadrupole mass spectrometer (Thermo TSQ-ultra) operated in positive electrospray ionization mode. Quantitation was accomplished by monitoring nucleoside-to-base ion transitions and generating standard curves for each nucleoside using the stable isotope dilution internal standardization method.[1]

Immunization, hybridomas, and antibody production

Immunizations and monoclonal antibody production were performed in collaboration with Abcam (Burlingame, CA) as reported previously.[2] Primary immunizations utilized ac4C-KLH conjugates (0.5 mg), while booster immunizations utilized ac4C-KLH (2x 0.25 mg) and ac4C OVA (0.25 mg). After immunizations, blood samples were taken to determine antibody titer by evaluating rabbit serum for specific detection of ac4C-containing RNA probes via dot blot. One rabbit was chosen for hybridoma development. After fusion, supernatants were tested for the presence of antibodies specific for ac4C by dot blot, and further evaluated for utility in RNA immunoprecipitation experiments. Optimal hybridomas were cloned by limiting dilution in 96-well plates, and those secreting preferred antibodies were chosen for production.

Scheme S1: Synthesis of ac4CTP.

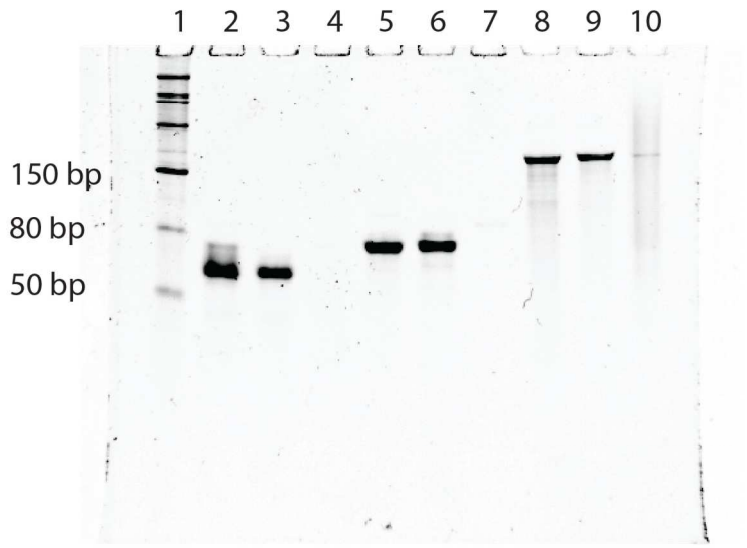


Synthesis of ac4CTP.

Ac4CTP was synthesized using a modification of a previously published protocol [3]. Briefly, tributylammonium pyrophosphate (73 mg, 0.16 mmol) and 2-chloro-1,3,2,-benzodioxaphosphorin-4-one (20 mg, 0.1 mmol) were dissolved separately in anhydrous DMF. Tributylamine (0.3 mL) was added to the solution of tributylammonium, and this entire solution was then added via syringe to the stirring 2-chloro-1,3,2,-benzodioxaphosphorin-4-one. To this mixture was added a solution of N4-acetylcytidine (23 mg, x 0.08 mmol) in anhydrous DMF. Care was taken to add the N4-acetylcytidine dropwise over a period of 5 min. The reaction was stirred for 2 hr. A solution of iodine (1 mL, 0.02 M in THF/pyridine, Glen Research) was added to the flask and the reaction was allowed to stir for an additional 30 min. Finally, 5 mL of deionized water was added to the flask and the reaction was allowed to stir for an additional 1.5 hr. Formation of ac4CTP was monitored by LC-MS. Pure ac4CTP was isolated by ethanol precipitation followed by HPLC purification on an Agilent 1260 system equipped with a Gemini 10u C18 250 X 21.2 mm column. Buffer A: 20 mM triethylammonium acetate (TEAAc, [pH 7.1]); Buffer B: 50% acetonitrile in 20 mM TEAAc [pH 7.1]; gradient of 0% to 40% buffer B over 30 minutes with a flow rate of 10 mL/min at a detection wavelength of 250 nm. Yield (9.69 mg, 23%). ESI-MS (positive mode): $[M+H]^+$ calculated: 525.2, [M+H]+ found: 526.0. ESI-MS (negative mode): $[M]^-$ calculated: 524.4, found: 524.1. $[M+Na]^-$ calculated: 548.2, found: 548.1. $[M+2Na]^-$ calculated: 570.4, found: 570.1. $[M+3Na]^-$ calculated: 593.0, found: 592.6. λ_{max} = 249 nm, 300 nm.

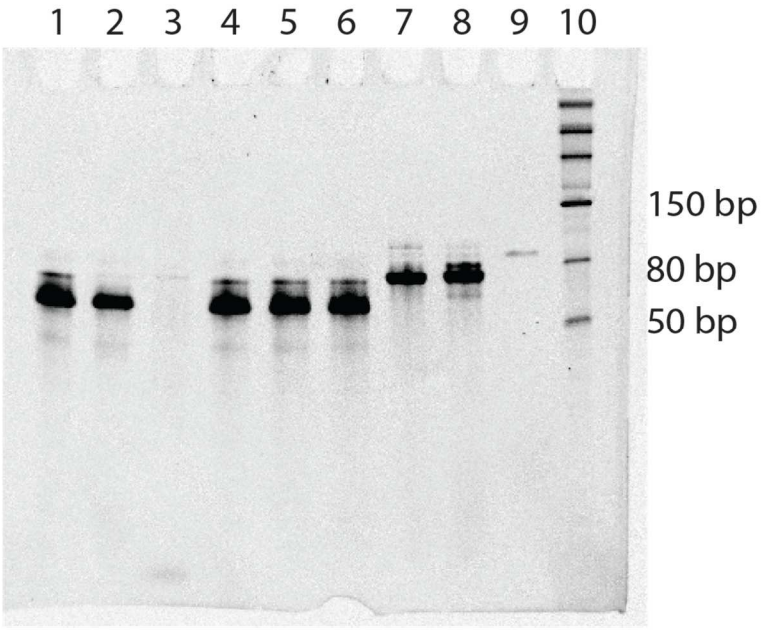
Full gel images for Figure 3 and Figure S5.

Full gel for Figure 3



Lane 1: ladder
Lane 2: IVT reaction, 18S rRNA template, CTP
Lane 3: IVT reaction, 18S rRNA template, ac4CTP
Lane 4: IVT reaction, 18S rRNA template, no CTP
Lane 5: IVT reaction, E. coli tRNA Met template, CTP
Lane 6: IVT reaction, E. coli tRNA Met template, ac4CTP
Lane 7: IVT reaction, E. coli tRNA Met template, no CTP
Lane 8: IVT reaction, mouse beta-globin template, CTP
Lane 9: IVT reaction, mouse beta-globin template, ac4CTP
Lane 10: IVT reaction, mouse beta-globin template, no CTP

Full gel for Figure S4



Lane 1: IVT reaction, 18S rRNA template, CTP
Lane 2: IVT reaction, 18S rRNA template, ac4CTP
Lane 3: IVT reaction, 18S rRNA template, no CTP
Lane 4: IVT reaction, 18S rRNA template, CTP, 37 degrees, 1 hr
Lane 5: IVT reaction, 18S rRNA template, CTP, 37 degrees, 1 hr, 50 mM hydroxylamine
Lane 6: IVT reaction, 18S rRNA template, CTP, 60 degrees, 1 hr, 50 mM hydroxylamine
Lane 7: IVT reaction, E. coli tRNA Met template, CTP
Lane 8: IVT reaction, E. coli tRNA Met template, ac4CTP
Lane 9: IVT reaction, E. coli tRNA Met template, no CTP
Lane 10: ladder

References:

1. Basanta-Sanchez, M.; Temple, S.; Ansari, S. A.; D'Amico, A.; Agris, P. F., Attomole quantification and global profile of RNA modifications: Epitranscriptome of human neural stem cells. *Nucleic Acids Res.* **2016,** *44*, e26.
2. Huang, Y.; Gu, B.; Wu, R.; Zhang, J.; Li, Y.; Zhang, M., Development of a rabbit monoclonal antibody group against Smads and immunocytochemical study of human and mouse embryonic stem cells. *Hybridoma* **2007,** *26*, 387-391.
3. Caton-Williams, J.; Hoxhaj, R.; Fiaz, B.; Huang, Z., Use of a novel 5'-regioselective phosphitylating reagent for one-pot synthesis of nucleoside 5'-triphosphates from unprotected nucleosides. *Curr. Protoc. Nucleic Acid Chem.* **2013,** *Chapter 1*, Unit 1 30.