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(57) Abrégé/Abstract:

Provided herein is a method for efficiently capping RNA in vitro. In some embodiments the capping reaction may be done at high temperature using Vaccinia capping enzyme or a variant thereof. In other embodiments, the capping reactions may comprise a capping enzyme from a large virus of amoeba, e.g., Faustovirus, mimivirus or mousmou virus, or a variant thereof. Compositions and kits for practicing the method are also provided.

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Abstract:

Provided herein is a method for efficiently capping RNA *in vitro*. In some embodiments the capping reaction may be done at high temperature using Vaccinia capping enzyme or a variant thereof. In other embodiments, the capping reactions may comprise a capping enzyme from a large virus of amoeba, *e.g.*, Faustovirus, mimivirus or moumou virus, or a variant thereof. Compositions and kits for practicing the method are also provided.

ENZYMATIC RNA CAPPING METHOD

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Provisional Application No. 62/890,821 filed August 23, 2019,
5 which is hereby incorporated, in its entirety, into this application by this reference.

BACKGROUND

The addition of a cap to uncapped synthetic RNA is important for efficient protein expression in many eukaryotic cells. Moreover, uncapped RNAs (at least RNAs that have a 5'-triphosphate) are reported to activate
10 the innate immune response (Pichlmair, et al., Science 2006 314: 997-1001; Diamond, et al., Cytokine & Growth Factor Reviews, 2014 25: 543-550). As such, it is highly desirable to add a cap to synthetic RNA in many therapeutic applications (e.g., protein replacement therapy as well as prophylactic or therapeutic vaccination).

Currently, two methods that are used to cap RNA. In the first method, synthetic RNAs (e.g., RNAs transcribed *in vitro*) are converted to capped RNAs using an RNA capping enzyme. In the other method (which is
15 generally referred to as "co-transcriptional capping") cap analogs such as anti-reverse cap analog (ARCA) and capped dinucleotides are added to the *in vitro* transcription reaction. In the co-transcriptional methods, the cap is co-transcriptionally incorporated into RNA molecules during *in vitro* transcription.

Compared to the co-transcriptional capping method, the enzymatic RNA capping method can achieve higher yields of capped RNA. However, enzymatic RNA capping reactions are quite inefficient and, as such,
20 either large amounts of enzyme are used, or the capped RNA must be purified from the uncapped RNA. Further, the efficiency of enzymatic RNA capping reactions (expressed as a percentage of capped RNA after the completion of a capping reaction) can vary from one RNA sequence to another, a difference that is generally attributed to RNA structure (see, e.g., Fuchs, RNA. 2016 22: 1454-66).

Therefore, there is still a need for a more efficient way to add cap to synthetic RNAs.

SUMMARY

This disclosure provides, among other things, a method for efficiently capping RNA *in vitro*. In some embodiments, a method may comprise contacting (i) an RNA sample comprising an uncapped target RNA, (ii) an RNA capping enzyme comprising an amino acid sequence that is at least 90% identical to (e.g., at least 95%
30 identical to) SEQ ID NOS:1, 7 or 20, (iii) guanosine triphosphate (GTP) or modified GTP, (iv) a buffering agent, and optionally (v) a methyl group donor at a temperature in the range of 40°C-60°C, to form (e.g., to efficiently form) a capped target RNA. The efficiency determined by yield of capped RNA (50%)/enzyme concentration (nM) may be improved by at least 2-fold or at least 3-fold compared to the capping efficiency of the enzyme at

37°C. An uncapped target RNA optionally may be free of modified nucleotides or may comprise one or more modified nucleotides (*e.g.*, pseudouridine).

In some embodiments, a method may comprise contacting (i) an RNA sample comprising an uncapped target RNA, (ii) a single-chain RNA capping enzyme that has RNA triphosphatase (TPase), guanylyltransferase (GTase) and guanine-N7 methyltransferase (N7 MTase) activities, (iii) GTP or modified GTP, (iv) a buffering agent, and optionally (v) methyl group donor at a temperature in the range of 37°C -60°C, to form (*e.g.*, to efficiently form) a capped target RNA. A single-chain RNA capping enzyme (*e.g.*, RNA capping enzymes from of giant viruses such as Faustovirus, Mimivirus or Moumouvirus) may comprise: (a) an amino acid sequence at least 90% identical to (*e.g.*, at least 95% identical to) (x) SEQ ID NO:2, (y) SEQ ID NO:3, and/or (z) SEQ ID NO:4 or (b) an amino acid sequence at least 90% identical to (*e.g.*, at least 95% identical to) (x) SEQ ID NO:5 and/or (y) SEQ ID NO:6. For example, RNA capping enzymes of giant viruses such as Faustovirus, Mimivirus or Moumouvirus may comprise an amino acid sequence at least 90% identical to (*e.g.*, at least 95% identical to) (a) SEQ ID NO:2, (b) SEQ ID NO:3, (c) SEQ ID NO:4, (d) SEQ ID NO:5, and/or (e) SEQ ID NO:6. RNA capping enzymes from Faustovirus (which are examples of single-chain RNA capping enzymes) may comprises an amino acid sequence that is at least 90% identical to (a) SEQ ID NO:7, (b) SEQ ID NO:8, (c) SEQ ID NO:9, (d) SEQ ID NO:10, (e) SEQ ID NO:11 and/or (f) SEQ ID NO:12.

In some embodiments, an uncapped target RNA may be at least 200 nt in length (*e.g.*, at least 300 nt, at least 500 nt or at least 1,000 nt) and may encode a polypeptide such as a therapeutic protein or vaccine. Target RNA having secondary structure including therapeutic RNA can be capped more efficiently using method of this disclosure. Efficiency may be defined as yield of capped RNA (50%) /enzyme concentration (nM). In any embodiment, a method may comprise contacting at a first temperature (*e.g.*, 37°C-60°C) and increasing or decreasing the temperature (*e.g.*, to 37°C-60°C) wherein the second temperature differs from the first temperature. For example, a method may include contacting at a first temperature of 37°C for 1 to 120 minutes and increasing the temperature to 45°C or 50°C for 1 to 120 minutes. A method, in some embodiments, may include increasing or decreasing the temperature to a third temperature of 37°C-60°C for 1 to 120 minutes. A capping method, in some embodiments, may produce (*e.g.*, may co-transcriptionally produce) more than 70% Cap0 RNA *in vitro* in one hour or less.

In some embodiments, the components and/or combinations thereof may be RNase-free and contacting may optionally further comprise (v) one or more RNase inhibitors.

The uncapped RNA may be synthesized using solid-phase oligonucleotide synthesis chemistry or by transcribing a DNA template using a polymerase (*e.g.*, T7 RNA polymerase or Hi-T7 RNA polymerase) in an *in vitro* transcription reaction, for example, by contacting a DNA template encoding the uncapped target RNA and the polymerase.

In any embodiment, contacting may further comprise contacting SAM and/or a cap 2'O methyltransferase enzyme (2'OMTase).

Contacting, in any embodiment, may comprise contacting (i), (ii), (iii), (iv) and (v) in a single location, for example, in a single microfluidics surface, a single reaction tube or other reaction vessel.

Also provided herein is a composition comprising an uncapped target RNA, a single-chain RNA capping enzyme that has TPase, GTase and N7 MTase activities, GTP and a buffering agent. In some embodiments, a composition may have a temperature in the range of 37°C -60°C. In some embodiments, a composition may be RNase-free and may optionally comprise one or more RNase inhibitors. In some embodiment, a single-chain RNA capping enzyme may comprise: (a) amino acid sequences that are at least 90% identical to (x) SEQ ID NO:2, (y) SEQ ID NO:3, and/or (z) SEQ ID NO:4 or (b) an amino acid sequence that is at least 90% identical to (x) SEQ ID NO:5 and/or (y) SEQ ID NO:6. A single-chain RNA capping enzyme may comprise an amino acid sequence that is at least 90% identical to the RNA capping enzyme of Faustovirus D5b (SEQ ID NO:7), Faustovirus E12 (SEQ ID NO:8), Faustovirus ST1 (SEQ ID NO:9), Faustovirus LC9 (SEQ ID NO:10), mimivirus (SEQ ID NO:11) or mousmavirus (SEQ ID NO:12). In some embodiments, a composition may further comprise a DNA template, a polymerase (*e.g.*, a bacteriophage polymerase) and ribonucleotides, for transcribing the RNA. In some embodiments, a composition may further comprise SAM and/or a cap 2'OMTase. In some embodiments, a single uncapped target RNA may be at least 200 nt in length (at least 300 nt, at least 500 nt or at least 1,000 nt) and may encode a polypeptide such as a therapeutic protein or vaccine.

Also provided is a kit. In some embodiments, a kit may comprise a single-chain RNA capping enzyme that has TPase, GTase and N7 MTase activities, wherein the enzyme is in a storage buffering agent; and a concentrated reaction buffering agent. In some embodiments, a kit may further include a DNA template, a polymerase and ribonucleotides, for transcribing the RNA. In some embodiments, a kit may further include SAM and/or a cap 2'OMTase. Examples of the methods, compositions and kits that utilize methyl transferases may also include SAM in the reaction mix. A single-chain RNA capping enzyme may comprise: (a) an amino acid sequence at least 90% identical to (x) SEQ ID NO:2, (y) SEQ ID NO:3, and/or (z) SEQ ID NO:4 or (b) an amino acid sequence at least 90% identical to (x) SEQ ID NO:5 and/or (y) SEQ ID NO:6. The single-chain RNA capping enzyme may comprises an amino acid sequence that is at least 90% identical to the RNA capping enzyme of Faustovirus D5b (SEQ ID NO:7), Faustovirus E12 (SEQ ID NO:8), Faustovirus ST1 (SEQ ID NO:9), Faustovirus LC9 (SEQ ID NO:10), mimivirus (SEQ ID NO:11) or mousmavirus (SEQ ID NO:12).

In some embodiments, an RNA capping enzyme may an amino acid sequence that is at least 90% identical to SEQ ID NO:20. In some embodiments, an RNA capping enzyme fusion may comprise (a) an amino acid sequence that is at least 90% identical to SEQ ID NO:7 and (b) an amino acid sequence that is at least 90% identical to positions 1419 to 1587 of SEQ ID NO:20.

BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1 shows the predicted secondary structure of the model RNA molecules used. RNA1 was designed to have the least secondary structure at the 5' end. RNA2, RNA3 and RNA4 were designed to exhibit the same predicted minimum free energy of unfolding (MFE) while containing a blunt end, one-base or two-base overhang at their 5' ends. The secondary structures and minimum free energy of unfolding at 37°C and 45°C were calculated using the RNAFold webserver (Vienna University, Wien, Austria). The modeled RNA molecules have the following sequences: RNA1: SEQ ID NO:13, RNA2: SEQ ID NO:14, RNA3: SEQ ID NO:15, RNA4: SEQ ID NO:16.

FIGURE 2 shows the RNA capping activity of H3C2 and VCE on RNA1 at different reaction temperatures. The amount of m7Gppp-capped RNA is a measure of RNA capping activity of the enzymes. Each bar represents the result of an average of 4 independent experiments, with the error bars indicating the standard deviation. As shown in the figure, both H3C2 and VCE exhibit maximum RNA capping activity at 45°C. While VCE capping activity dropped precipitously at 50°C and higher, H3C2 retained significant capping activity at 50°C, 52°C and 55°C.

FIGURES 3A-3B shows the RNA capping activity at 37°C, 45°C, 50°C and 55°C as a function of capping enzyme concentration. FIGURE 3A shows activity of VCE. FIGURE 3B shows activity of H3C2. See Table 1 for a quantitative comparison of the capping activity under each condition.

FIGURES 4A-4E RNA capping activity of putative RNA capping enzymes on a 150 nt RNA at various temperatures. Lanes 1 through 6 show capping reactions using products of *in vitro* translation of AMN83561 (H3C2) (lane 1), AIB52055 (lane 2), SME65026 (lane 3), SMH63629 (lane 4), AAV50651 aa 1-668 (mimivirus capping enzyme N-terminal region) (lane 5), or VP_007354410 (Moumouvirus capping enzyme) (lane 6). In lane 7, purified H3C2 (20 nM) was used. The ribonucleotide sequence of the 150 nt RNA used in these experiments is: GGGAGUCUUCGCCGAGAGGGCCAUCGCCAGUUGCCGCAACCUGUGGGAAUUUCUCUUCAGUUU AUCCGGAUGCUCAACGGUGACUUUAAUUCGGUAUCUUUCUGAAUUUCUUACCGACUUCAGCG AGCUCGACAAUGCUCUUCUUU (SEQ ID NO:17). FIGURE 4A shows capping activity at 37°C. FIGURE 4B shows capping activity at 45°C. FIGURE 4C shows capping activity at 50°C. FIGURE 4D shows capping activity at 55°C. FIGURE 4E shows capping activity at 60°C.

FIGURE 5 shows the conserved regions that exhibit 90% or higher sequence identity among the Faustovirus D5b, E12, ST1 and LC9 (SEQ ID NOS: 7-10) capping enzyme amino acid sequences mapped to AMH83561 (H3C2). The conserved regions are designated as Fausto_CR_01, Fausto_CR_03 and Fausto_CR_05. Functional domains of H3C2 capping enzyme, namely TPase, GTase and N7 MTase, are indicated

FIGURE 6 shows the conserved regions that exhibit 90% or higher sequence identity among the *Acanthamoeba polyphaga* mimivirus (AAV50651, SEQ ID NO:11) and *Acanthamoeba polyphaga* moumouvirus capping enzyme amino acid sequences mapped to YP_007354410 (*Acanthamoeba polyphaga*

moumouvirus capping enzyme; moumou CE) (SEQ ID NO:12). The conserved regions are designated as moumou_CR_03 (SEQ ID NO:6) and moumou_CR_04 (SEQ ID NO:7). Functional domains of moumou CE, namely TPase, GTase and N7 MTase, are indicated in this alignment with dashed lines above the aligned sequences. The conserved regions are highlighted.

FIGURE 7 shows that RNA capping reactions performed at 45°C facilitate efficient capping of hairpin RNAs. At 37°C (white bars), both H3C2 and VCE efficiently capped RNA1, which has the least 5' secondary structure (see FIGURE 1). RNA2, RNA3 and RNA4, which have stable 5' secondary structures at 37°C (see FIGURE 1), were either not capped or capped to a low extent at 37°C (white bars). At 45°C (grey bars), however, H3C2 capped all four RNAs efficiently. VCE efficiently capped RNA1 and RNA2 and partially capped RNA3 and RNA4 at 45°C (grey bars).

FIGURE 8 shows that RNA capping reactions performed at 45°C facilitate efficient capping of long RNA. Increasing concentrations of H3C2 or VCE were incubated with 400 nM of *fluc* RNA (1.7 kb) in the presence of 0.5 mM GTP and 0.1 mM SAM in 10 µL of 1x RNA capping buffering agent (50 mM Tris-HCl, 5 mM KCl, 1 mM MgCl₂, 1 mM DTT, pH 8) at 37°C or 45°C for 30 minutes. The first 24 nt of *fluc* RNA was then cleaved off by oligo-guided RNase H cleavage. The extent of capping in the cleaved 24 nt fragments was analyzed by electrophoresis over 15% urea polyacrylamide gels. The band intensity was quantified and used to calculate the fraction capped under each condition. The calculated values were graphed against enzyme concentration and fitted to a modified Hill equation to derive the enzyme concentration at which 50% capping was achieved (Cap₅₀). For H3C2, the Cap₅₀ values were 33.6 nM and 0.96 nM at 37°C or 45°C, respectively. For VCE, the Cap₅₀ values were 103 nM and 2.97 nM at 37°C or 45°C, respectively. For both H3C2 and VCE, reacting at 45°C substantially decreased the quantity of enzyme needed to cap 50% of the substrate RNA than at 37°C.

FIGURE 9 shows that RNA capping reactions performed at 45°C facilitate more efficient capping of long RNA other than *fluc* RNA than at 37°C. 100 nM of H3C2 or VCE was incubated with 500 nM of *cluc* RNA (1.8 kb) or CFTR RNA (4.0 kb) in the presence of 0.5 mM GTP and 0.1 mM SAM in 10 µL of 1x RNA capping buffering agent (50 mM Tris-HCl, 5 mM KCl, 1 mM MgCl₂, 1 mM DTT, pH 8) at 37°C or 45°C for 30 min. Defined 5' fragments were cleaved by using Thermostable RNase H (New England Biolabs, Ipswich, MA) and purified using a combination of AMPure® XP Beads (Beckman Coulter, Indianapolis, IN) and streptavidin Dynabeads® (Thermo Fisher Scientific, Waltham, MA). Intact mass analysis of the purified 5' fragments were carried out by Novatia LLC (Newtown, PA) using a high-resolution LC/MS workflow. The fraction of capped RNA was calculated using the mass intensity of capped and uncapped species.

FIGURE 10 shows that high reaction temperatures enhance enzymatic activity of Vaccinia virus RNA cap 2'O methyltransferase (MTase). 5 µM of chemically synthesized Cap-0 25-nt RNA (m7Gppp25mer) was allowed to react with 200 µM of SAM in the presence of 100 U of VacciniaVaccinia cap 2'O MTase (New England

Biolabs, Ipswich MA) in 20 μ L of 1x RNA capping buffering agent at the designated temperature for 30 minutes. The reactions were stopped by heating at 70°C for 10 minutes. The RNA was purified from the reaction components, digested into nucleosides and cap structures and analyzed by UPLC. More Cap-1 RNA can be generated by carrying out the reaction at 45°C and 50°C than at 37°C.

FIGURE 11 shows that “one-pot” generation of Cap-1 structure is more efficient at 45°C than at 37°C. In this experiment, 5 μ M of a chemically synthesized 5’ triphosphate ribonucleotide oligo (ppp25mer) was incubated with 200 U of Vaccinia virus cap 2’OMTase in the presence of 50 nM H3C2 or VCE, 1 mM GTP and 0.2 mM SAM in 40 μ L of 1x RNA capping buffering agent at 37°C or 45°C for 30 minutes. The relative quantity of the reactants and products were derived from direct LC/MS analysis. As shown here, performing the one-pot enzymatic reaction at 45°C generates more Cap-1 structured RNA than at 37°C.

FIGURE 12 shows that single-step capped RNA synthesis for a 1.7 kb *fluc* transcript using commercially available enzymes and recommended reaction temperature does not generate significant amount of Cap-0 or Cap-1 RNA. Reactions were carried out at 37°C for 1 hour using the indicated components at recommended enzyme concentrations (see Example 7 for details). Transcription output was measured using Qubit RNA BR kit after DNase I treatment. Products of capped RNA synthesis reactions were analyzed by LC/MS. Fraction of transcript containing different 5’ groups were estimated using mass intensity of the corresponding masses. Datapoints are average of triplicate experiments unless indicated otherwise.

FIGURE 13 shows a single-step method to synthesize capped *fluc* transcript using high concentration of H3C2 capping enzyme and increased temperature. Single-step capped RNA synthesis were carried out at 37 or 45°C for 1 hour using T7 RNA polymerase with 0.5 μ M H3C2 RNA capping enzyme. Fraction of ppp-, pp-, Gppp- and m7Gppp-capped RNA was estimated using capillary electrophoresis. Datapoints are average of duplicate experiments.

FIGURE 14 shows a single-step Cap-0 or Cap-1 RNA synthesis using high capping enzyme concentration at 45 or 50°C. Products of capped RNA synthesis reactions were analyzed by LC/MS. Fraction of transcript containing different 5’ group was estimated using mass intensity of the corresponding masses. Datapoints are average of triplicate experiments unless indicated otherwise.

FIGURE 15 shows a single-step Cap-0 RNA synthesis using H3C2:T7 fusion protein at 37°C or 45°C. The fusion protein generated 98% Cap-0 transcript at 45°C in 1 hour. Products of capped RNA synthesis reactions were analyzed by LC/MS. Fraction of transcript containing different 5’ group was estimated using mass intensity of the corresponding masses. Datapoints are average of duplicate experiments unless indicated otherwise.

FIGURE 16 shows that VCE and H3C2 capping enzyme efficiently cap the 1.8 kb *cluc/A120* transcript. 500 nM of unmodified or pseudouridine *cluc/A120* transcript was incubated with 100 nM of H3C2 or VCE in the presence of 0.5 mM GTP and 0.1 mM SAM in 10 mL of 1x RNA capping buffering agent (50 mM Tris-HCl, 5 mM KCl, 1 mM MgCl₂, 1 mM DTT, pH 8) at 37°C for 30 min. The capping reactions were analyzed by the

RNase H/intact LC/MS workflow as described in EXAMPLE 9. VCE and H3C2 generated 68.4% or 100% m7Gppp-capped *cluc/A120* transcript containing pseudouridine, respectively. Notably, both capping enzymes cap a larger fraction of the pseudouridine-containing *cluc/A120* transcript than the uridine counterpart.

FIGURE 17 shows the Cap-1 formation efficiency of a larger RNA quantity. A total of 250 pmol (145 μ g) of ~1800 nt *cluc/A120* *in vitro* transcript was capped in a 500 μ L reaction containing 1x RNA capping buffering agent (50 mM Tris-HCl, pH 8.0, 5 mM KCl, 1 mM MgCl₂, 1 mM DTT), 0.5 mM GTP, 0.1 mM SAM, 10 U/ μ L of Vaccinia cap 2'O methyltransferase and 500 nM of H3C2 at 45°C for 1 h. The efficiency of Cap-1 formation was assessed by the RNase H/intact LC/MS as described in Example 10. Under these reaction conditions, 54.8% of the pseudouridine containing *cluc/A120* transcript was Cap-1 whereas 47.7% of the uridine counterpart was Cap-1. Optimization of reaction conditions (as described in EXAMPLE 10) may improve the yield of Cap-1 formation. Results are average of duplicate experiments.

FIGURE 18 shows that the Cap-1 *cluc/A120* transcript are functionally active *in vivo*. The Cap-1 *cluc/A120* transcript containing uridine or pseudouridine and transfected to HEK293 cells. The translation efficiency of the capped *cluc/A120* transcript was measured as relative luciferase activity from HEK293 cells transfected with the capped *cluc/A120* transcript in relation to uncapped *cluc/A120* transcript four hours after transfection. The luciferase assay shows that the capped transcripts containing rU and pseudouridine exhibited luciferase activity 10 or 20-fold, respectively, over uncapped controls, indicating that the RNAs capped with H3C2 and Vaccinia 2'O methyltransferase at 45°C were functionally active.

FIGURE 19 shows that multiple temperature steps can improve the transcription output and capping efficiency of single-vessel capped RNA synthesis. Single-vessel capped RNA synthesis reactions using T7 RNA polymerase paired with VCE or H3C2 capping enzyme were performed for a 1.7 kb *fluc* transcript as described in Example 11, where reactions were incubated at 37°C for 30 min, followed by a second 30-minute incubation at temperatures between 28°C and 50°C. The transcription output was evaluated using DNase I/Qubit method. Fraction capped was estimated by the RNase H/Klenow fill-in and urea PAGE method. Samples that showed the highest fraction capped were subjected to intact LC/MS analysis. For VCE, the m7Gppp- transcript forming efficiency peaked at second reaction temperature of 45°C, whereas for H3C2, the m7Gppp-forming efficiency peaked at second reaction temperature of 50°C. T7 RNAP transcription output, on the other hand, peaked at 45°C when paired with either VCE or H3C2, reflecting the low transcription activity at the lower and upper ends of the tested temperatures (28°C and 50°C, respectively). Hence, tandem temperature steps of 37°C for 30 min followed by 45°C for 30 min yielded the optimal balance of transcription output and capping efficiency in this example. Modifications to reaction conditions such as the reaction time, reaction temperature and the number of temperature steps may further improve the transcription yield and fraction of m7G-capped transcript.

DETAILED DESCRIPTION

Aspects of the present disclosure can be further understood in light of the embodiments, section headings, figures, descriptions and examples, none of which should be construed as limiting the entire scope of the present disclosure in any way. Accordingly, the claims set forth below should be construed in view of the full breadth and spirit of the disclosure.

Each of the individual embodiments described and illustrated herein has discrete components and features which can be readily separated from or combined with the features of any of the other several embodiments without departing from the scope or spirit of the present teachings. Any recited method can be carried out in the order of events recited or in any other order which is logically possible. Disclosed reaction conditions may be varied, including, but not limited to, reaction temperature, reaction duration, reaction components (*e.g.*, enzymes, substrates) and/or reactant concentrations.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. Still, certain terms are defined herein with respect to embodiments of the disclosure and for the sake of clarity and ease of reference.

Sources of commonly understood terms and symbols may include: standard treatises and texts such as Kornberg and Baker, *DNA Replication*, Second Edition (W.H. Freeman, New York, 1992); Lehninger, *Biochemistry*, Second Edition (Worth Publishers, New York, 1975); Strachan and Read, *Human Molecular Genetics*, Second Edition (Wiley-Liss, New York, 1999); Eckstein, editor, *Oligonucleotides and Analogs: A Practical Approach* (Oxford University Press, New York, 1991); Gait, editor, *Oligonucleotide Synthesis: A Practical Approach* (IRL Press, Oxford, 1984); Singleton, et al., *Dictionary of Microbiology and Molecular biology*, 2d ed., John Wiley and Sons, New York (1994), and Hale & Markham, the *Harper Collins Dictionary of Biology*, Harper Perennial, N.Y. (1991) and the like.

As used herein and in the appended claims, the singular forms “a” and “an” include plural referents unless the context clearly dictates otherwise. For example, the term “a protein” refers to one or more proteins, *i.e.*, a single protein and multiple proteins. It is further noted that the claims can be drafted to exclude any optional element. As such, this statement is intended to serve as antecedent basis for use of such exclusive terminology as “solely,” “only” and the like in connection with the recitation of claim elements or use of a “negative” limitation.

Numeric ranges are inclusive of the numbers defining the range. All numbers should be understood to encompass the midpoint of the integer above and below the integer *i.e.*, the number 2 encompasses 1.5-2.5. The number 2.5 encompasses 2.45-2.55 *etc.* When sample numerical values are provided, each alone may represent an intermediate value in a range of values and together may represent the extremes of a range unless specified. *e.g.*

In the context of the present disclosure, “buffering agent”, refers to an agent that allows a solution to resist changes in pH when acid or alkali is added to the solution. Examples of suitable non-naturally occurring

buffering agents that may be used in the compositions, kits, and methods of the invention include, for example, Tris, HEPES, TAPS, MOPS, tricine, or MES.

In the context of the present disclosure, “capping” refers to the enzymatic addition of a Nppp- moiety onto the 5' end of an RNA, where N a nucleotide such as is G or a modified G. A modified G may have a methyl group at the N7 position of the guanine ring, or an added label at the 2 or 3 position of the ribose, and, in some embodiments, the label may be an oligonucleotide, a detectable label such as a fluorophore, or a capture moiety such as biotin or desthiobiotin, where the label may be optionally linked to the ribose of the nucleotide by a linker, for example. See, *e.g.*, WO 2015/085142. A cap may have a Cap-0 structure, a Cap-1 structure or a cap 2 structure (as reviewed in Ramanathan, Nucleic Acids Res. 2016 44: 7511–7526), depending on which enzymes and/or whether SAM is present in the capping reaction.

In the context of the present disclosure, “DNA template” refers to a double stranded DNA molecule that is transcribed in an *in vitro* transcription reaction. DNA templates have a promoter (*e.g.*, a T7, T3 or SP6 promoter) recognized by the RNA polymerase upstream of the region that is transcribed.

In the context of the present disclosure, “fusion” refers to two or more polypeptides, subunits, or proteins covalently joined to one another (*e.g.*, by a peptide bond). For example, a protein fusion may refer to a non-naturally occurring polypeptide comprising a protein of interest covalently joined to a reporter protein. Alternatively, a fusion may comprise a non-naturally occurring combined polypeptide chain comprising two proteins or two protein domains joined directly to each other by a peptide bond or joined through a peptide linker.

In the context of the present disclosure, “*in vitro* transcription” (IVT) refers to a cell-free reaction in which a double-stranded DNA (dsDNA) template is copied by a DNA-directed RNA polymerase (typically a bacteriophage polymerase) to produce a product that contains RNA molecules copied from the template.

In the context of the present disclosure, “Faustovirus RNA capping enzyme” refers to a single-chain RNA capping enzyme capable of capping RNA (*e.g.*, having detectable TPase, GTase and N7 MTase activity) including, for example, enzymes having at least 90% identity to faustovirus D5b (SEQ ID NO:7), Faustovirus E12 (SEQ ID NO:8), Faustovirus ST1 (SEQ ID NO:9), or Faustovirus LC9 (SEQ ID NO:10). “H3C2 RNA capping enzyme”, “H3C2 capping enzyme” or “H3C2” refers to the RNA capping enzyme from faustovirus D5b (SEQ ID NO:7). Unless expressly stated otherwise, Faustovirus RNA capping enzymes may be interchangeable with one another for purposes of illustrations and examples disclosed herein with similar properties, effects, and/or benefits.

In the context of the present disclosure, “H3C2 fusion” refers to a bifunctional enzyme capable of synthesizing an RNA from a template polynucleotide and capable of capping RNA, the fusion comprising an RNA polymerase (*e.g.*, T7 RNA polymerase, T3 RNA polymerase, SP6 RNA polymerase and a Faustovirus RNA capping enzyme arranged on the N-terminal end or the C-terminal end relative to the polymerase. An H3C2 fusion may further comprise a leader and/or a linker (*e.g.*, between the polymerase and the H3C2). An H3C2

fusion may have, for example, an amino acid sequence having at least 80%, at least 85%, at least 90%, at least 93%, at least 96%, at least 97%, at least 98% or at least 99% identity to SEQ ID NO:20. An H3C2:T7 RNA polymerase fusion is an example of an H3C2 fusion.

In the context of the present disclosure, “H3C2 variant” refers to H3C2, H3C2 fusions, and enzymes capable of capping RNA, in each case, comprising an amino acid sequence having at least 80%, at least 85%, at least 90%, at least 93%, at least 96%, at least 97%, at least 98% or at least 99% identity to SEQ ID NO:7, 8, 9, 10, 11, and/or 12.

In the context of the present disclosure, “modified nucleotides” (including references to modified NTP, modified ATP, modified GTP, modified CTP, and modified UTP) refers to any noncanonical nucleoside, nucleotide or corresponding phosphorylated versions thereof. Modified nucleotides may include one or more backbone or base modifications. Examples of modified nucleotides include dI, dU, 8-oxo-dG, dX, and THF. Additional examples of modified nucleotides include the modified nucleotides disclosed in U.S. Patent Publication Nos. US20170056528A1, US20160038612A1, US2015/0167017A1, and US20200040026A1. Modified nucleotides may include naturally or non-naturally occurring nucleotides.

In the context of the present disclosure, “non-naturally occurring” refers to a polynucleotide, polypeptide, carbohydrate, lipid, or composition that does not exist in nature. Such a polynucleotide, polypeptide, carbohydrate, lipid, or composition may differ from naturally occurring polynucleotides polypeptides, carbohydrates, lipids, or compositions in one or more respects. For example, a polymer (*e.g.*, a polynucleotide, polypeptide, or carbohydrate) may differ in the kind and arrangement of the component building blocks (*e.g.*, nucleotide sequence, amino acid sequence, or sugar molecules). A polymer may differ from a naturally occurring polymer with respect to the molecule(s) to which it is linked. For example, a “non-naturally occurring” protein may differ from naturally occurring proteins in its secondary, tertiary, or quaternary structure, by having a chemical bond (*e.g.*, a covalent bond including a peptide bond, a phosphate bond, a disulfide bond, an ester bond, and ether bond, and others) to a polypeptide (*e.g.*, a fusion protein), a lipid, a carbohydrate, or any other molecule. Similarly, a “non-naturally occurring” polynucleotide or nucleic acid may contain one or more other modifications (*e.g.*, an added label or other moiety) to the 5'-end, the 3' end, and/or between the 5'- and 3'-ends (*e.g.*, methylation) of the nucleic acid. A “non-naturally occurring” composition may differ from naturally occurring compositions in one or more of the following respects: (a) having components that are not combined in nature, (b) having components in concentrations not found in nature, (c) omitting one or components otherwise found in naturally occurring compositions, (d) having a form not found in nature, *e.g.*, dried, freeze dried, crystalline, aqueous, and (e) having one or more additional components beyond those found in nature (*e.g.*, buffering agents, a detergent, a dye, a solvent or a preservative). All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual

publication, patent, or patent application was specifically and individually indicated to be incorporated by reference.

In the context of the present disclosure, “RNA sample” or “sample” refers to a composition that may or may not comprise a target RNA. For example, an RNA sample may be known to include or suspected of including such target RNA and/or an RNA sample may be a composition to be evaluated for the presence of a target RNA. An RNA sample may comprise a naturally occurring target RNA (*e.g.*, extracted from a cell, tissue, or organism), a target RNA produced by *in vitro* transcription, and/or a chemically synthesized RNA.

In the context of the present disclosure, “single-chain RNA capping enzyme” refers to a capping enzyme in which a single polypeptide chain includes the TPase, GTase and N7 MTase activities. Faustovirus, mimivirus and mousmouvirus capping enzymes are examples of single-chain RNA capping enzymes. H3C2 fusions are additional examples of single-chain RNA capping enzymes. VCE is a heterodimer and, as such, is not a single-chain RNA capping enzyme.

In the context of the present disclosure, “single uncapped RNA target species” refers to a mixture of target RNA molecules that have essentially the same sequence. Transcripts made by *in vitro* transcription and RNA oligonucleotides made by solid-phase synthesis are examples single uncapped RNA target species. It is recognized that a certain amount of the RNA products in such a mixture may be truncated. Single uncapped RNA target species may sometimes contain modified nucleotides (*e.g.*, noncanonical nucleotides that are not found in nature). Preparations of RNA from a cell contain a complex mixture of naturally occurring RNA molecules having different sequences; such preparations do not contain only targeted uncapped RNA species but also contain a wide variety of non-target RNAs. In some embodiments, the targeted uncapped RNA species is a single species of RNA.

In the context of the present disclosure, “target RNA” refers to a polyribonucleotide of interest. A polyribonucleotide may be or comprise a therapeutic RNA or precursor thereof (*e.g.*, an uncapped precursor of a capped therapeutic RNA). A target RNA may arise from cellular transcription or *in vitro* transcription. A target RNA may be present in a mixture, for example, an *in vitro* transcription reaction mixture, a cell, or a cell lysate. A target RNA may be uncapped. If desired or required, a target RNA may be contacted with a decapping enzyme, for example, as a co-treatment with or pre-treatment before capping.

In the context of the present disclosure, “uncapped” refers to an RNA (a) that does not have a cap and (b) that can be used as a substrate for a capping enzyme. Uncapped RNA typically has a tri- or di-phosphorylated 5' end. RNAs transcribed *in vitro* have a triphosphate group at the 5' end.

In the context of the present disclosure, “variant” refers to a protein that has an amino acid sequence that is different from a naturally occurring amino acid sequence (*i.e.*, having less than 100% sequence identity to the amino acid sequence of a naturally occurring protein) but that is at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98% or at least 99% identical to the naturally occurring amino acid sequence.

Provided herein are a variety of *in vitro* RNA capping methods. Some embodiments of the method are based, in part, on the discovery that VCE of SEQ ID NO:1 is significantly more active at 45°C as compared to 37°C (see FIGURE 2 and Tables 1 and 2, below), particularly for RNAs that have secondary structure. For example, when used to add a cap to an *in vitro* transcribed RNA that encodes a protein, the same amount of capped product can be produced at 45°C using less than a thirtieth (1/30th) of the amount of enzyme, as compared to 37°C (see Table 6). In addition, incubation at the reaction at 45°C allows the VCE to efficiently add a cap to uncapped RNA oligonucleotides that have secondary structure at the 5' end (*e.g.*, a 5' end that is predicted to base pair with another sequence in the molecule to produce a blunt end or a 3' or 5' overhang of 1 or 2 nucleotides, as exemplified in FIGURE 1). Such RNAs are very inefficiently capped using the VCE at 37°C (see FIGURE 9). In some embodiments therefore, the method may comprise incubating a reaction mix comprising RNA, VCE or a variant thereof, GTP or modified GTP, SAM and a buffering agent at a temperature in the range of 42°C-47°C, *e.g.*, 44°C -46°C to efficiently add a cap structure to the uncapped RNA target. These embodiments of the method are particularly useful for RNAs having or predicted to have secondary structure, such as RNAs over 200 nt in length transcribed *in vitro*, and RNA oligonucleotides that have secondary structure at the 5' end.

Other embodiments of the method are based in part on the discovery that the RNA capping enzyme from faustovirus D5b (SEQ ID NO:7, an example of an H3C2 capping enzyme and referenced here by the shorthand "H3C2") is capable of capping RNA significantly more efficiently than the VCE at almost all temperatures tested (see FIGURE 2 and Tables 1 and 6, below) and, like the VCE, can be used to efficiently add a cap to an *in vitro* transcribed RNA at 45°C. For example, the same amount of capped product can be produced at 45°C using less than a thirtieth (1/30th) of the amount of faustovirus D5b (H3C2), as compared to 37°C (see Table 6). In addition, incubating the reaction at 45°C allows the faustovirus D5b (H3C2) to efficiently add a cap to uncapped RNA oligonucleotides that have secondary structure at the 5' end. Such RNAs are very inefficiently capped using the faustovirus D5b (H3C2) 37°C (see FIGURE 9). Capping enzymes from other giant viruses of the amoebas, including faustovirus ST1, faustovirus LC9, faustovirus E12, mimivirus, and moomouvirus were also tested and found to be active at higher temperatures. As such, in some embodiments, the method may comprise incubating a reaction mix comprising a sample comprising RNA, a single-chain capping enzyme, GTP or modified GTP and a buffering agent at a temperature in the range of 37°C -60°C, *e.g.*, 37°C -42°C, 42°C -47°C, 47°C -52°C or 52°C -60°C to efficiently add a cap structure to the uncapped RNA target. *in vitro* These embodiments of the method are particularly useful for RNAs that have or predicted to have secondary structure such as RNAs over 200 nt in length transcribed *in vitro*, and RNA oligonucleotides that have secondary structure at the 5' end.

More efficient capping of the RNA substrate may support capping with less enzyme added to a capping reaction, producing more capped RNA (as a percentage of the RNA in the reaction) using the same amount of enzyme, terminating the reaction earlier, and/or capping RNAs that have secondary structure at the 5' end more efficiently.

Disclosed reaction conditions may be varied, including, without limitation, reaction temperature, reaction duration, reaction component concentrations (*e.g.*, SAM, inorganic pyrophosphatase, NTPs, transcript template), and enzymes (*e.g.*, capping enzymes, polymerases, and fusions thereof). For example, an H3C2:T7 RNA polymerase fusion protein may further improve the efficiency of Cap-0 RNA synthesis in terms of fraction of Cap-0 transcripts and transcription output. In addition, inclusion of a cap 2'O methyltransferase, such as Vaccinia cap 2'O methyltransferase, in the reactions can generate Cap-1 transcript at high efficiency. Depending on which enzyme is used and the other components in the reaction mix, disclosed methods may be used to make RNAs that have a Gppp cap, a 7-methylguanylate cap (*i.e.*, a m7Gppp cap, or "cap-0"), or an RNA that has an m7Gppp cap that has addition modifications in the first and/or second nucleotides in the RNA (*i.e.*, "cap-1" and "cap-2"; see Fechter J. Gen. Vir. 2005 86: 1239–49). For example, if there is no SAM in the reaction mix then RNAs that have a Gppp cap may be produced. If the reaction mix comprises SAM in addition to the capping enzyme, then cap-0 RNA may be produced. If the reaction mix comprises other enzymes, *e.g.*, cap 2' OMTase in addition to SAM, then cap-1 and/or cap-2 RNA may be produced. A reaction mix may comprise other components in addition to those explicitly described above.

In some embodiments, uncapped RNA in the reaction mix may be prepared by solid-phase oligonucleotide synthesis chemistry (see, *e.g.*, Li, et al J. Org. Chem. 2012 77: 9889–9892), in which case the RNA in the sample may be may have a length in the range of 10-500 bases, *e.g.*, 20-200 bases). In other embodiments, uncapped RNA in the reaction mix may be prepared in a cell free *in vitro* transcription (IVT) reaction in which a double-stranded DNA template that contains a promoter for an RNA polymerase (*e.g.*, a T7, T3 or SP6 promoter) upstream of the region that is transcribed is copied by a DNA-directed RNA polymerase (typically a bacteriophage polymerase) to produce a product that contains RNA molecules copied from the template. In either embodiment, the RNA sample that is capped in the present method contains a single species of RNA (either the synthetic oligonucleotide or the transcript). In addition, the reaction mix may be RNase-free and may optionally comprise one or more RNase inhibitors. The RNA in the sample may contain a non-natural sequence of nucleotides and in some embodiments, may contain non-naturally occurring nucleotides. In some embodiments, the *in vitro* transcription reaction may employ a thermostable variant of the T7, T3 and SP6 RNA polymerase (see, *e.g.*, PCT/US2017/013179 and US application serial no. 15/594,090). In these embodiments, the RNAs may be transcribed at a temperature of greater than 44°C (*e.g.*, a temperature of at least 45°C, at least 50°C, at least 55°C or at least 60°C, up to about 70°C or 75°C) in order to reduce the immunogenicity of the RNA (see, *e.g.*, WO 2018/236617). In some cases, the uncapped RNA may be capped immediately after it is made, *e.g.*, by adding an RNA capping enzyme and GTP/modified GTP, as needed to the *in vitro* transcription reaction after the reaction has run its course. In some embodiments, the RNA made in an *in vitro* transcription reaction may be purified prior to capping.

In some embodiments, the RNA in the sample may be a therapeutic RNA. In these embodiments, the product of the present method may be used without purification of the capped RNA from the uncapped RNA. In these embodiments, the product of the present reaction may be combined with a pharmaceutical acceptable excipient to produce a formulation, where “pharmaceutical acceptable excipient” is any solvent that is compatible with administration to a living mammalian organism via transdermal, oral, intravenous, or other administration means used in the art. Examples of pharmaceutical acceptable excipients include those described for example in US 2017/0119740. The formulation may be administered *in vivo*, for example, to a subject, examples of which include a human or any non-human animal (*e.g.*, mouse, rat, rabbit, dog, cat, cattle, swine, sheep, horse or primate). Depending on the subject, the RNA (modified or unmodified) can be introduced into the cell directly by injecting the RNA or indirectly via the surrounding medium. Administration can be performed by standardized methods. The RNA can either be naked or formulated in a suitable form for administration to a subject, *e.g.*, a human. Formulations can include liquid formulations (solutions, suspensions, dispersions), topical formulations (gels, ointments, drops, creams), liposomal formulations (such as those described in: US 9,629,804 B2; US 2012/0251618 A1; WO 2014/152211; US 2016/0038432 A1). The cells into which the RNA product is introduced may be *in vitro* (*i.e.*, cells that cultured *in vitro* on a synthetic medium). Accordingly, the RNA product may be transfected into the cells. The cells into which the RNA product is introduced may be *in vivo* (cells that are part of a mammal). Accordingly, the introducing may be done by administering the RNA product to a subject *in vivo*. The cells into which the RNA product is introduced may be present *ex vivo* (cells that are part of a tissue, *e.g.*, a soft tissue that has been removed from a mammal or isolated from the blood of a mammal).

Synthesis of large amounts of uniformly capped mRNA transcripts in a cost-effective and streamlined manner that is scalable to support multi-gram synthesis may be desired or required to manufacture mRNA for therapeutic applications. Current approaches to mRNA manufacturing use costly mRNA cap analogs in *in vitro* transcription reactions. Alternatively, separate reactions are required to produce 5'- triphosphate RNA by *in vitro* transcription followed by mRNA capping using enzymes – a more complex process that is harder to scale. A single-step *in vitro* synthesis of capped RNA using T7 RNA polymerase and a capping enzyme may streamline the manufacturing process. Single-vessel reactions with both enzymes may reduce or remove otherwise prohibitive costs of synthetic mRNA cap analogs and may reduce the complexity of scaling this workflow to support multi-gram (and beyond) synthesis.

Methods

Provided herein are methods for efficiently producing capped RNA. In some embodiments, methods include thermoactive Hi-T7 RNA polymerase and H3C2 RNA capping enzyme and unexpectedly thermoactive Vaccinia mRNA cap 2'O methyltransferase. Surprisingly, reaction temperatures substantially higher than 37°C enable single reaction capped mRNA synthesis. In some embodiments, RNA transcription and capping activities

may be performed in a single reaction at higher temperatures (*e.g.*, 40°C to 60°C). For example, RNA polymerases and individual capping enzymes with or without Vaccinia cap 2'O methyltransferase may be used simultaneously in capped RNA synthesis reactions. Combining RNA transcription and capping activities may be achieved, for example, with fusion proteins comprising H3C2, a single-subunit RNA capping enzyme, and T7 RNA polymerase or Hi-T7 RNA polymerase. Methods described herein can achieve high levels of Cap-0 or Cap-1 RNA synthesis.

A capping method, in some embodiments, may comprise contacting an RNA polymerase (*e.g.*, a T7 RNA polymerase, a thermoactive Hi-T7 RNA polymerase and/or an H3C2 fusion), a polynucleotide (*e.g.*, DNA or RNA) encoding a target RNA, a capping enzyme (*e.g.*, VCE, H3C2, an H3C2 fusion), NTPs, a buffering agent, optionally in the presence or absence of SAM. Nucleoside triphosphates (NTPs) may include unmodified ATP, modified ATP (m6ATP, m1ATP), unmodified CTP, modified CTP (*e.g.* m5CTP), unmodified GTP, modified GTP, unmodified UTP, modified UTP (*e.g.*, pseudouridine triphosphate, m1pseudouridin triphosphate), NTPs containing 2'O methylation, and/or combinations thereof. In some embodiments, a capping method may comprise contacting a capping enzyme (*e.g.*, VCE, H3C2, an H3C2 fusion), a target RNA, and guanosine triphosphate (GTP), and/or modified GTP. A capping method may comprise, in some embodiments, contacting a capping enzyme (*e.g.*, VCE, H3C2, an H3C2 fusion), a target RNA, and guanosine triphosphate (GTP) and/or modified GTP, an O-methyl transferase (*e.g.*, thermoactive Vaccinia mRNA cap 2'O methyltransferase), in the presence or absence of SAM.

Contacting, according to some embodiments, may be performed in a single location (*e.g.*, in a single step), for example, on any surface (*e.g.*, plate or bead) or in any vessel (*e.g.*, tube, flask, vial, column, container, bioreactor or other space). In some embodiments, contacting may comprise contacting some or all the subject elements in any desired order or concurrently. Contacting, in some embodiments, may comprise contacting some or all the subject elements in the presence of a buffering agent. For example, contacting may include contacting, in any order or concurrently, a capping enzyme (*e.g.*, VCE, H3C2, an H3C2 fusion), a target RNA, GTP, and a buffering agent in the presence or absence of SAM. In some embodiments, contacting may further comprise contacting at a temperature of 40°C to 60°C (*e.g.*, at 40°C, 42°C, 44°C, 45°C, 46°C, 48°C, 50°C, 52°C, 54°C, 55°C, 56°C, 58°C, or 60°C) for any desired period of time, for example, 1 minute to 120 minutes (*e.g.*, 1 minute, 5 minutes, 10 minutes, 15 minutes, 20 minutes, 25 minutes, 30 minutes, 45 minutes, 60 minutes, 75 minutes, 90 minutes, 105 minutes, or 120 minutes). In any of the embodiments disclosed herein, contacting may further comprise contacting at a first temperature of 25°C to 60°C and cooling or warming to a second temperature of 25°C to 60°C, different from the first temperature, wherein, optionally, at least one of the first temperature or second temperature is not less than 40°C. A first temperature may be selected to produce, stabilize and/or retain a desired amount of uncapped target RNA and a second temperature may be selected to produce, stabilize and/or retain a desired amount capped target RNA. Each temperature may be maintained for any desired period of time

(*e.g.*, 1 minute to 120 minutes). For example, a first temperature may be 37°C for a first period of 1 to 30 minutes and a second temperature may be 28°C, 32°C, 37°C, 45°C, or 50°C for a second period of 1 to 30 minutes.

Contacting, in any of the disclosed embodiments, may include placing one item in direct contact with another, placing two items together on a surface or in a vessel in sufficient proximity that they may physically and/or chemically interact with or without further agitation or mixing. Contacting may include the initial act of placing one item in direct contact with or proximity to another and/or maintaining conditions that permit or favor the two items to contact each other.

A method, in some embodiments, may comprise synthesizing RNA (*e.g.*, directed or undirected by a template) *in vitro* to produce an uncapped target RNA and capping the target RNA (*e.g.*, in single-step reactions). Synthesizing RNA from a template may comprise, for example, contacting an RNA polymerase (*e.g.*, a T7 RNA polymerase, a thermoactive Hi-T7 RNA polymerase and/or an H3C2 fusion) with the template (*e.g.*, an RNA template or a DNA template) and one or more NTPs to produce an uncapped target RNA, a buffering agent, and optionally in the presence or absence of SAM. Nucleoside triphosphates (NTPs) may include unmodified ATP, modified ATP (m6ATP, m1ATP), unmodified CTP, modified CTP (*e.g.* m5CTP), unmodified GTP, modified GTP, unmodified UTP, modified UTP (*e.g.*, pseudouridine triphosphate, m1pseudouridin triphosphate), NTPs containing 2'O methylation, and/or combinations thereof. Capping a target RNA may comprise, for example, contacting H3C2 (*e.g.*, H3C2 or an H3C2 fusion) with a target RNA and guanosine triphosphate (GTP) and/or a modified GTP to produce a capped target RNA. In some embodiments, a capping method may comprise contacting a target RNA with an H3C2 fusion at a temperature of 25°C to 60°C. A capping method may comprise, in some embodiments, contacting a target RNA with a decapping enzyme to remove an existing cap and/or assure that it is uncapped.

Capping may comprise, for example, contacting a capping enzyme (*e.g.*, VCE, H3C2, an H3C2 fusion) with a target RNA, an O-methyltransferase (*e.g.*, thermoactive Vaccinia mRNA cap 2'O methyltransferase), SAM, guanosine triphosphate (GTP), modified GTP, and/or a buffering agent. For example, capping may comprise contacting a capping enzyme, a target RNA, GTP (optionally, with or without modified GTP), and optionally a buffering agent. Capping may comprise contacting, in a single vessel (*e.g.*, in a single step), a capping enzyme, a target RNA, GTP (optionally, with or without modified GTP), an O-methyltransferase, SAM, and optionally a buffering agent. Capping may comprise capping a pre-existing target RNA and/or synthesizing and capping a target RNA in a single vessel (*e.g.*, in a single step), for example, by contacting a target RNA template (DNA or RNA) encoding the target RNA, a polymerase (*e.g.*, a T7 RNA polymerase, a thermoactive Hi-T7 RNA polymerase and/or an H3C2 fusion), NTPs (optionally including or excluding one or more modified NTPs), a capping enzyme (*e.g.*, VCE, H3C2, an H3C2 fusion), and/or a buffering agent.

Compositions

A variety of compositions related to the disclosed methods are also provided. In some embodiments, a composition (*e.g.*, a reaction mix) may comprise a target RNA (*e.g.*, in an RNA sample) and/or a DNA template encoding a target RNA. In some embodiments, a composition may comprise a single-chain RNA capping enzyme having TPase, GTase and N7 MTase activities, GTP, SAM and a buffering agent. A composition may have a temperature in the range of 37°C -60°C, *e.g.*, 37°C-42°C, 42°C -47°C, 47°C -52°C or 52°C-60°C. In some embodiments, a composition may be free of RNase activity as a result of RNases being inhibited, inactivated or absent. For example, an RNase-free composition may comprise one or more RNase inhibitors. In some embodiments, a composition may further comprise a DNA template operably linked to a promoter, a bacteriophage polymerase that initiates transcription at the promoter, and NTPs, for transcribing the RNA. In some embodiments, a composition may include a cap 2'OMTase. A single-chain RNA capping enzyme may comprise (a) an amino acid sequence at least 90% identical to SEQ ID NO:2, 3, or 4, or (b) an amino acid sequence at least 90% identical to SEQ ID NO:5 or 6. A composition, according to some embodiments, may comprise an H3C2 fusion comprising, in N-terminal to C-terminal order, (a) Faustovirus RNA capping enzyme (*e.g.*, an H3C2 RNA capping enzyme) and an RNA polymerase or (b) an RNA polymerase and an H3C2 RNA capping enzyme, wherein the H3C2 fusion optionally may further comprise a leader (*e.g.*, operably positioned within the fusion) and/or a linker positioned between the H3C2 and the RNA polymerase. In some embodiments, any or all of the components referenced in this paragraph may be combined or otherwise included in a single container. Components, for example, components in a single container, may be present in dry (*e.g.*, anhydrous and/or lyophilized) form. A container with one or more of the referenced components may also contain an aqueous medium.

Kits

Also provided by this disclosure are kits for practicing the subject method, as described above. In some embodiments, the kit may contain any one or more of the components listed above. For example, a kit may contain a single-chain RNA capping enzyme that has TPase, GTase and N7 MTase activities, wherein the enzyme is in a storage buffering agent and a reaction buffering agent (*e.g.*, which may be a 5x or 10x concentrated reaction buffering agent). In some embodiments, the kit may comprise a bacteriophage polymerase and NTPs, for transcribing the RNA from a DNA template. The kit may additionally comprise SAM and a cap 2'OMTase. As would be apparent from the discussion above, the enzyme may comprise (a) an amino acid sequences that are at least 90% identical to SEQ ID NOS: 2, 3, or 4, or (b) an amino acid sequence that is at least 90% identical to SEQ ID NOS: 5 or 6.

The various components of the kit may be present in separate containers or certain compatible components may be pre-combined into a single container, as desired. In addition to above-mentioned components, the subject kits may further include instructions for using the components of the kit to practice the

subject methods, i.e., to instructions for capping an RNA.

EXAMPLES

All reagents are available from New England Biolabs (Ipswich, MA) and/or the indicated supplier.

Example 1: RNA capping activity of H3C2 and VCE at high temperatures

10 nM of purified VCE or H3C2 RNA capping enzyme was incubated with 500 nM RNA1 (FIGURE 1) containing a fluorescent group (FAM) at its 3' end in the presence of 0.5 mM GTP and 0.1 mM SAM in 10 μ L of 1x RNA capping buffering agent (50 mM Tris-HCl, 5 mM KCl, 1 mM MgCl₂, 1 mM DTT, pH 8.0) at temperatures ranged from 23°C to 65°C for 30 minutes. The reactions were quenched by adding 1 μ L 50 mM EDTA followed by heat-inactivation at 70°C for 10 minutes. The quenched reactions were analyzed by capillary electrophoresis using an Applied Biosystems 3730xl Genetic Analyzer. The peak area corresponding to the m7Gppp-, unmethylated Gppp-, uncapped pp- and ppp-RNA1 were quantified and used to calculate fraction of m7Gppp- at the end of the 30-minute capping reaction. In FIGURE 2, each bar represents an average of 4 independent experiments. Error bars represent the standard deviation of the 4 replicates. It is clear that at low enzyme concentration (10 nM), both H3C2 and VCE exhibited highest capping activity at 45°C whereas H3C2 retains higher capping activity than VCE at 50°C and 55°C.

To better quantify the RNA capping activity of VCE and H3C2 at different reaction temperatures, dilutions of the enzymes were incubated under the same reaction conditions at 37°C, 45°C, 50°C or 55°C, respectively, for 30 minutes. The calculated values of fraction m7Gppp-RNA1 were graphed against enzyme concentration and fitted to a Hill equation derivative to derive the enzyme concentration at which 50% capping was achieved (Cap50). As showed in FIGURES 3A-3B and Table 1, it requires 3.06 nM, 0.94 nM, 4.08 nM and 30.00 nM of VCE to convert 50% of 0.5 μ M of ppp-RNA1 to m7Gppp-RNA1 at 37°C, 45°C, 50°C or 55°C, respectively, in 30 minutes. Furthermore, at 50°C and below, 400 nM or more VCE caps >90% of 0.5 μ M RNA in 30 min. For H3C2, 0.69 nM, 0.67 nM, 2.15 nM or 21.3 nM of enzyme can convert 50% of ppp-RNA1 into m7Gppp-RNA1 at 37°C, 45°C, 50°C or 55°C, respectively, in 30 minutes. In addition, 50 nM of H3C2 effectively caps >90% of ppp-RNA1 at up to 55°C in 30 minutes.

Table 1. RNA capping activity of VCE and H3C2 at various temperatures expressed in Cap50 values in nM (A) or milli-unit per μ l of reaction (B).

A	Cap50 (nM)				Fold improvement 45°C vs 37°C
	37°C	45°C	50°C	55°C	
VCE	3.06	0.94	4.08	30.00	3.24

H3C2	0.69	0.67	2.15	21.30	1.03
Fold improvement H3C2 vs VCE	4.46	1.42	1.90	1.41	

B	Cap50 (mU/ μ L)				Fold improvement 45°C vs 37°C
Reaction temperature	37°C	45°C	50°C	55°C	
VCE	15.30	4.72	20.40	149.98	3.24
H3C2	3.43	0.67	10.75	1.03	1.03
Fold improvement H3C2 vs VCE	4.46	1.42	1.90	1.41	

Example 2: Identification and testing of H3C2 orthologs

Purified RNA capping enzyme H3C2 from faustovirus D5b (Genbank® accession AMN83561) was active up to 60°C under *in vitro* conditions. By sequence homology, several putative orthologs in other faustovirus strains were identified. It was found that the gene product of the putative RNA capping enzymes from other faustovirus strains and related giant virus *Acanthanomeba polyphaga* mimivirus and *Acanthanomeba polyphaga* mounouvirus were active in RNA capping up to 55-60°C. The ORF of the identified orthologous genes were synthesized and inserted into a T7 expression vector such that the expression of the target genes was under the control of T7 promoter. The T7 expression cassettes were amplified by PCR using Q5® High Fidelity 2x Master Mix (New England Biolabs, Ipswich, MA) according to manufacturer's recommendations. The amplified T7 expression cassettes were purified using the Monarch® PCR & DNA cleanup kit (New England Biolabs, Ipswich, MA) according to manufacturer's instructions. The purified T7 express cassettes were subjected to *in vitro* transcription/translation using PURExpress® *in vitro* Protein Synthesis Kit (New England Biolabs, Ipswich, MA) according to manufacturer's instructions. The PURExpress reaction products, containing the gene products with the indicated Genbank accession numbers (FIGURES 4A-4E), were used in RNA capping assays. Briefly, 50 μ L RNA capping reactions made up of 2 μ L of PURExpress product, 0.4 μ M of a 150 nt *in vitro* transcript RNA, 4 μ M GTP, 0.1 mM SAM, and a trace amount of 32 P- α -GTP in 1x RNA capping buffering agent (50 mM Tris-HCl, 5 mM KCl, 1 mM MgCl₂, 1 mM DTT, pH 8.0) were assembled on ice. The reactions were divided into 5 equal parts, each of which were incubated at the indicated temperatures for 30 minutes. The reactions were stopped by adding 10 μ L of 2x RNA Loading Dye (New England Biolabs, Ipswich, MA) to the

reactions and analyzed by denaturing polyacrylamide electrophoresis and autoradiography. Capping activity is indicated by radiographic signal that migrates at the position of the substrate RNA as indicated. As shown in FIGURES 4A-4E, putative RNA capping enzymes from faustovirus ST1 (Genbank accession SME65026; lanes 3) and faustovirus LC9 (SMH63629; lanes 4), the N-terminal fraction of mimivirus capping enzyme (aa 1-668 of Genbank accession AAV50651; Benarroch *et al*, 2008; lanes 5) and mousmouvirus capping enzyme (Genbank accession YP_007354410; lanes 6) cap the 150 mer RNA at temperatures up to 55°C, whereas RNA capping enzyme from Faustovirus D5b (Genbank accession AMN83561; H3C2; lanes 1), Faustovirus E12 (Genbank accession AIB52055; lanes 2) and purified H3C2 (lanes 7) cap the 150 mer RNA at temperatures up to 60°C.

Example 3: Sequence analysis

Sequence alignment analysis revealed that three 60 aa- or 111 aa-long regions exhibit 90% or higher amino acid sequence identity among the four Faustovirus RNA capping enzymes that are active up to 55°C (FIGURE 5). Conserved region Fausto_CR_01 spans 60 aa within the TPase domain of H3C2. Fausto_CR_03 spans 111 aa at the N-terminal of the guanosine N7 methyltransferase domain. Fausto_CR_05 spans 60 aa at the C-terminal side of the guanosine N7 methyltransferase domain of H3C2. The amino acid sequence of Fausto_CR_01 (which corresponds to amino acids 43-102 of the H3C2 sequence) is FDKLKPDGEITTTMRVSNADGMAREITFGGGVK TGEMFVKKQNICVFDVVDIFS YKVA VS (SEQ ID NO:2). The amino acid sequence of Fausto_CR_03 (which corresponds to amino acids 537-647 of the H3C2 sequence) is GFYGN NYKIASDIY LNYI DVFNFDDLWKYNPGYFEKNKSDIYIAPNKYRRYLIKSLFNKYIKNAKWVID AAAGRGADLHLYKAECVENLLAIDIDPTAISELIRRRNEITG (SEQ ID NO:3). The amino acid sequence of Fausto_CR_05 (which corresponds to amino acids 778-873 of the H3C2 sequence) is KYSIKRLYDSDKLTKTGQKIAVLLPMSG EMKEEPLCNIKNIISMAR KMGLDLVESANFSV (SEQ ID NO:4).

The following tables summarize the sequence identities between the amino acid sequences of Faustovirus capping enzymes.

Table 2

	AMN83561 Faustovirus D5b	AIB52055 Faustovirus E12	SME65026 Faustovirus ST1	SMH63629 Faustovirus LC9_partial
AMN83561 Faustovirus D5b	100%	71%	71%	77%
AIB52055 Faustovirus E12	71%	100%	97%	70%
SME65026 Faustovirus ST1	71%	97%	100%	69%

SMH63629 Faustovirus LC9_partial	77%	70%	69%	100%
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Table 3

	AMN83561 Faustovirus D5b	AIB52055 Faustovirus E12	SME65026 Faustovirus ST1	SMH63629 Faustovirus LC9_partial
Fausto_CR_01	92%	98%	98%	92%
Fausto_CR_03	90%	99%	99%	90%
Fausto_CR_05	93%	97%	97%	92%

FIGURE 6 shows an alignment of the various Faustovirus RNA capping enzyme sequences, showing the conserved domains as well as the sequences of the TPase, GTase and MTase regions.

Sequence alignment analysis also revealed that two 67 aa- or 111 aa-long regions exhibit 90% or higher sequence identity between *Acanthanomeba polyphaga* mimivirus and *Acanthanomeba polyphaga* moudouvirus RNA capping enzymes that are active up to 55°C (FIGURE 7). Conserved region moudou_CR_03 (which corresponds to amino acids 576-642 of the moudouvirus sequence) spans 67 aa between the GTase and guanosine N7 methyltransferase domains. Moudou_CR_04 (which corresponds to amino acids 672-782 of the moudouvirus sequence) spans 111 aa at the N-terminal of the guanosine N7 methyltransferase domain. The amino acid sequence of moudou_CR_03 is INDNTVVEFIFDNFKIDMDDPYKWIP IRTRYDKTESVQKYHKKYGNL HIANRIWKTITNPITEDII (SEQ ID NO:5). The amino acid sequence of moudou_CR_04 is YYQKNTSNAAGMRAFNFIKSNMITTYCKDGDGVLDIGCGRGGDLIKFIHAGIEEYV GIDIDNNGLYVINDSAFNRYKNLKKTIKNIPPMTFINADARGLFNLEAQEKILP (SEQ ID NO:6).

The following tables summarize the sequence identities between the amino acid sequences of the mimivirus and moudouvirus RNA capping enzymes.

Table 4

	AAV50651.1 mimivirus	YP_007354410 Moudouvirus
AAV50651.1 mimivirus	100%	61%
YP_007354410 Moudouvirus	61%	100%

Table 5

	AAV50651.1 mimivirus	YP_007354410 Moudouvirus
Moudou_CR_03	91%	90%

Moumou_CR_04	91%	92%
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FIGURE 8 shows an alignment of the mimivirus and moumou virus sequences, showing the conserved domains as well as the sequences of the TPase, GTase and MTase regions.

5 **Example 4: RNA capping reactions at 45°C enhance capping efficiency on short model hairpin RNAs**

The short RNAs illustrated in FIGURE 1 were designed as described here to evaluate capping efficiency. A control RNA (RNA1) was designed to have a shorter hairpin structure and an unstructured 5' end with a MFE of -1.5 kcal/mol at 37°C or -0.73 kcal/mol at 45°C (FIGURE 1). RNA2, RNA3 and RNA4 were designed to form a stable hairpin structure with a theoretical minimum free energy of unfolding (MFE) of -7.2 kcal/mol at 37°C or -5.4 kcal/mol at 45°C (RNAFold server rna.tbi.univie.ac.at). RNA2, RNA3 and RNA4 were further designed to have a blunt end, a one-base or a two-base overhang at their 5' end of the respective minimum free energy structure (FIGURE 1). The RNAs were produced by *in vitro* transcription using T7 RNA polymerase. For capping reactions, 50 nM of H3C2 or VCE was incubated with 500 nM of RNA substrates in the presence of 0.5 mM GTP and 0.1 mM SAM in 10 µL of 1x RNA capping buffering agent (50 mM Tris-HCl, 5 mM KCl, 1 mM MgCl₂, 1 mM DTT, pH 8.0) at 37°C or 45°C for 30 min. At 37°C, H3C2 capped 100% of RNA1, which is predicted to have an unstructured 5' end, and 22% of RNA4, which is predicted to have a two-base overhang structure at the 5' end (FIGURE 9). H3C2 was unable to cap RNA2 and RNA3 under these conditions. At 45°C, H3C2 capped 100% of all four RNA substrates. VCE was only able to cap 80% of RNA1 at 37°C. At 45°C, VCE capped 100% of RNA1 and RNA2 and approximately 60% of RNA3 and RNA4.

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Example 5: RNA capping reactions at 45°C enhance capping efficiency on long RNAs

A 1766 nt RNA containing the firefly luciferase gene (*flucfluc*) was produced by *in vitro* transcription using T7 RNA polymerase. 400 nM of *fluc* RNA was incubated with the indicated concentrations of H3C2 or VCE in the presence of 0.5 mM GTP and 0.1 mM SAM in 10 µL of 1x RNA capping buffering agent (50 mM Tris-HCl, 5 mM KCl, 1 mM MgCl₂, 1 mM DTT, pH 8) at 37°C or 45°C for 30 minutes. A final concentration of 2.5 µM of targeting oligo (TO) designed to direct RNase H to cleave out a 5' fragment of 25 nt was then added to each capping reaction, which was then heated at 80°C for 30 s to inactivate the capping enzymes and then cooled slowly to room temperature. Thermostable RNase H (New England Biolabs, Ipswich, MA) was added to each reaction at a final concentration of 0.5 U/µL, followed by incubation at 37°C for 1 hour. The RNase H reactions were then analyzed by electrophoresis through 15% urea polyacrylamide gels. The gels were stained using SYBR® Gold (Thermo Fisher Scientific, Waltham, MA) (according to manufacturer's instructions, and then scanned using an Amersham® Typhoon® RGB (GE Healthcare, Marlborough, MA) scanner using the Cy2 channel. The intensity of the capped and uncapped bands was normalized to that of the TO of the same lane. The

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normalized values were used to calculate the fraction of RNA capped under each condition. The calculated values were graphed against enzyme concentration and fitted to a Hill equation derivative to derive the enzyme concentration at which 50% capping was achieved (Cap50). For H3C2, the Cap50 values were 33.6 nM and 0.96 nM at 37°C or 45°C, respectively. For VCE, the Cap50 values were 103 nM and 2.97 nM at 37°C or 45°C, respectively (FIGURE 10 and Table 6 below). For both enzymes, substantially less enzyme was needed to achieve 50% capping when capping reactions were carried out at 45°C.

Table 6. RNA capping activity of VCE and H3C2 on 1.7 kb *fluc in vitro* transcript at 37°C and 45°C expressed in Cap50 values.

Reaction temperature	Cap50 (nM)		Fold improvement 45°C vs 37°C
	37°C	45°C	
H3C2	33.60	0.96	35.00
VCE	103.14	2.97	34.73
Fold improvement H3C2 vs VCE	3.07	3.09	

The activity of these enzymes can be expressed in milli-unit per μ L of reaction, as shown below:

Reaction temperature	Cap50 (mU/ μ L)		Fold improvement 45°C vs 37°C
	37°C	45°C	
H3C2	168.00	4.80	35.00
VCE	515.70	14.85	34.73
Fold improvement H3C2 vs VCE	3.07	3.09	

To show that the temperature-enhanced capping efficiency can be achieved on other long RNA molecules, the capping efficiency of H3C2 and VCE were studied using *in vitro* transcripts of cypridina luciferase (*cluc*; 1823 nt) and cystic fibrosis transmembrane receptor (CFTR; 4712 nt) by a mass spectrometry readout. Briefly, 0.5 μ M of *cluc* or CFTR *in vitro* transcripts were incubated with 100 nM of H3C2 or VCE in the presence of 0.1 mM SAM and 0.5 mM GTP in a reaction volume of 100 μ L at 37°C or 45°C for 30 minutes. The reactions were stopped by heating at 80°C for 30 seconds in the presence of 2.5 μ M of targeting oligo composed of 5' deoxynucleotides and 3' ribonucleotides and a TEG-desthiobiotin group. The reactions were allowed to cool down to 25°C at a rate of 0.1°C/s. The reactions were then subjected to RNase H cleavage by incubation with thermostable RNase H (New England Biolabs, Ipswich, MA) at a final concentration of 0.5 U/ μ L at 37°C for 1 hour. The 5' fragments of the RNase H cleavage reactions were then purified using size selection using

AMPure® XP Beads and target selection using streptavidin magnetic beads. Briefly, 100 µL of RNase H cleavage reactions were added to 200 µL of AMPure® XP Beads and incubated at room temperature for 5 minutes. The beads were then subjected to a magnetic field, and the clarified supernatant was retrieved and added to pre-cleared AMPure® XP beads derived from 200 µL of beads suspension. After incubating at room temperature for 5 min, the beads were subjected to a magnetic field. The clarified supernatant was added to pre-cleared beads derived from 200 µL of Dynabeads® MyOne Streptavidin C1. After washing 4 times with 200 µL of wash buffering agent (5 mM Tris, pH 7.5, 0.5 mM EDTA, 1 M NaCl), the bound RNA was eluted by incubating the clarified beads with 50 µL of biotin elution buffering agent (1 mM biotin, 5 mM Tris, pH 7.5, 0.1 M NaCl, 0.1 M NaCl) at 37°C for 1 hour. The eluted RNA was then analyzed by LC/MS to determine the relative quantity of the target masses, performed by external contractor Novatia LLC. The extent of RNA capping was assessed by the ratio of the mass intensity of the capped and uncapped RNA species.

As shown in FIGURE 11, both H3C2 and VCE exhibit more capping activity on cluc RNA and CFTR RNA at 45°C than at 37°C.

Example 6: Efficient one-pot enzymatic synthesis of Cap-1 structure on triphosphate RNA at increased temperature

To verify if Vaccinia virus cap 2'OMTase is active at elevated temperatures, a reaction containing 5 µM of a chemically synthesized Cap-0 RNA1 (25 nt) and 200 µM SAM in 20 µL of 1x RNA capping buffering agent (50 mM Tris-HCl, 5 mM KCl, 1 mM MgCl₂, 1 mM DTT, pH 8.0) were preheated at 37°C, 45°C or 50°C for 1 minute before 100 U of Vaccinia virus cap 2'OMTase (New England Biolabs, Ipswich, MA) was added. The reactions were allowed to proceed at the pre-heat temperature for 30 minutes and then were stopped by heating at 70°C for 10 minutes. The RNA was purified from the reaction components using the Oligo Clean-up and Concentration Kit (Norgen Biotek, Thorold, Canada). The purified RNA was then digested into nucleosides and cap structures by incubating with 2 µL of nucleotide digestion mix (New England Biolabs, Ipswich, MA) in 20 µL reactions containing nucleoside digestion mix reaction buffering agent (50 mM sodium acetate, pH 5.4, 1 mM ZnCl₂) at 37°C for 1 hour. The nucleoside digestion reactions were then analyzed with Agilent 1290 Infinity II UHPLC (Agilent Technologies, Santa Clara, CA) on a Waters XSelect™ HSS T3 XP column (Waters Corporation, Milford, MA) (2.1 × 100 mm, 2.5 µm) with a gradient mobile phase consisting of methanol and 10 mM potassium phosphate buffering agent (pH 7.0). As shown in FIGURE 12, more Cap-0 RNA was methylated at 45°C and 50°C than at 37°C.

To demonstrate one-pot enzymatic synthesis of Cap-1 structure on 5' triphosphate RNA at increased temperature, 5 µM of a chemically synthesized 5' triphosphate RNA1 was incubated with 200 U of Vaccinia virus cap 2'OMTase (final concentration of 5 U/µL) in the presence of 50 nM H3C2 or VCE, 1 mM GTP and 0.2 mM

SAM in 40 μ L reactions containing 1x RNA capping buffering agent (50 mM Tris-HCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, pH 8.0) at 37°C or 45°C for 30 minutes. The reactions were directly analyzed by LC/MS to determine the mass and the relative quantity of the masses. When paired with 50 nM of VCE, while 5 U/ μ L of 2'OMTase only generated ~50% of the Cap-01 RNA at 37°C, it generated 100% Cap-1 RNA at 45°C (FIGURE 13). When paired with 50 nM of H3C2, 5 U/ μ L Vaccinia 2'OMTase generated ~90% Cap-1 RNA at 37°C and 100% Cap-1 RNA at 45°C. (FIGURE 13). Therefore, performing the reactions at 45°C approximately doubled the yield of Cap-1 RNA in the presence of VCE and increased the yield of Cap-1 RNA in the presence of H3C2.

Example 7: Single-step Cap-0 and Cap-1 RNA synthesis using individual RNA polymerase, RNA capping enzymes and cap 2'O methyltransferase Cap-0Cap-1

A. Single Step capped RNA synthesis with Vaccinia capping enzyme is inefficient

As recommended by the manufacturer for the respective reactions, 5 U/ μ L of T7 RNA polymerase (New England Biolabs; Cat. No. M0251), Hi-T7 RNA polymerase (New England Biolabs; Cat. No. M0658), 1 U/ μ L of Vaccinia capping enzyme (New England Biolabs; Cat. No. M2080) and 5 U/ μ L of Vaccinia mRNA cap 2'O methyltransferase (New England Biolabs; Cat. No. M0366) were used as indicated for the transcription and capping of a 1.7 kb *fluc* transcript (SEQ ID NO:18). The reactions contained 1x T7 RNA polymerase buffering agent (40 mM Tris-HCl, pH 7.9, 10 mM NaCl, 1 mM DTT, 2 mM spermidine) when T7 RNA polymerase was used or 1x Hi-T7 RNA polymerase buffering agent (40 mM Tris-HCl, pH 7.9, 60 mM NaCl, 1 mM DTT, 2 mM spermidine) when Hi-T7 RNA polymerase was used. The reactions were supplemented with 19 mM $MgCl_2$ and 5 mM each of ATP, UTP, GTP, CTP and 0.5 mM SAM. The reactions were carried out at 37°C for 1 hour, the recommended reaction temperature and duration for T7 RNA polymerase and Vaccinia capping enzyme.

To assess the yield of *in vitro* transcription, after the 1-hour incubation, for each reaction, 1 μ L of the reaction mix was added to a 4- μ L solution containing 1x DNase I reaction buffering agent (10 mM Tris-HCl, pH 7.6, 2.5 mM $MgCl_2$, 0.5 mM $CaCl_2$) and 0.5 U/ μ L DNase I (New England Biolabs) and incubated at 37°C for 30 min. The DNase I reactions were then analyzed for total RNA concentration using Qubit RNA Broad Range kit (Thermo Fisher) according to manufacturer's instruction. To verify the transcript sizes and integrity, the DNase I reactions were analyzed using 2% E-Gel (Thermo Fisher) with images taken using a Typhoon RGB scanner (GE Healthcare). Results are shown as transcription output in FIGURE 14.

To analyze the extent of RNA capping, single-step capped RNA synthesis reactions were analyzed by RNase H cleavage followed by intact LC/MS analysis. Briefly, the single-step capped RNA synthesis reactions stopped by heating at 80°C for 30s in the presence of 2.5 μ M of targeting oligo (TO-1) composed of 5' deoxynucleotides and 3' ribonucleotides and a TEG-desthiobiotin group (SEQ ID NO:19). The reactions were allowed to cool down to 25°C at a rate of 0.1°C/s. The reactions were then subjected to RNase H cleavage by incubation with thermostable RNase H (New England Biolabs; Cat. No. M0523) at a final concentration of 0.5

U/ μ L at 37°C for 1 h. To facilitate the analysis of the 5' group using capillary electrophoresis, a FAM-labeled nucleotide was added to the 3' end of the RNase H cleavage fragment. Briefly, 5 μ L of the RNase H reaction was retrieved and added to 5 μ L of a solution containing 2x NEBuffer 2, 0.5 mM dATP, 0.05 mM FAM-12-dCTP (Perkin Elmer) and 0.25 U/ μ L DNA polymerase I large (Klenow) fragment (New England Biolabs). The reactions were incubated at 37°C for 1 hour. In some instances, a small fraction of the Klenow reactions were analyzed on urea PAGE to verify the success of capping. Briefly, 2 μ L of the Klenow reactions were retrieved and added to 8 μ L of 2x RNA loading dye (New England Biolabs). The mixtures were then analyzed by electrophoresis through a urea-15% polyacrylamide gel. Gel images were acquired by a Typhoon RGB scanner (GE Healthcare) using the Cy2 channel.

To analyze the 5' group status of the coupled transcription/capping reaction products using capillary electrophoresis or mass spectrometry, the Klenow reaction products (RNase H-cleavage products still annealed to the desthiobiotinylated targeting oligo) were then purified by size selection using AMPure® XP Beads (Thermo Fisher) and then selected using streptavidin magnetic beads. Briefly, 45 μ L of nuclease-free water was added to 5 μ L of RNase H cleavage reactions, which was then added to 100 μ L of NEBNext® Sample Purification Beads (New England Biolabs) and incubated at room temperature for 5 minutes. The beads were then placed next to a magnet for 2 minutes at room temperature. The clarified supernatant was retrieved and added to pre-cleared NEBNext® Sample Purification Beads derived from 100 μ L of beads suspension. After incubating at room temperature for 5 min, the beads were then placed next to a magnet for 2 minutes at room temperature. The clarified supernatant was added to pre-cleared beads derived from 50 μ L of Dynabeads® MyOne Streptavidin C1 (Thermo Fisher). After washing 4 times with 50 μ L of low salt wash buffering agent (5 mM Tris, pH 7.5, 0.5 mM EDTA, 60 mM NaCl), the bound RNA was eluted by incubating the clarified beads in 10 μ L of nuclease-free water. The eluted RNA was then analyzed by capillary electrophoresis on an Applied Biosystems 3130xl Genetic Analyzer (16 capillary array) or an Applied Biosystems 3730xl Genetic Analyzer (96 capillary array) using GeneScan® 120 LIZ dye Size Standard (Applied Biosystems). Reaction products were analyzed using PeakScanner software (Thermo Fisher Scientific) and an in-house software suite. Areas of peaks corresponding to the m7Gppp-, unmethylated Gppp-, uncapped pp- and ppp- RNaseH-cleaved transcript were quantified and used to calculate fraction of ppp-, pp-, Gppp- and m7Gppp- transcript in the capped RNA synthesis reactions.

To determine the relative quantity of m7GpppGm- (Cap-1), m7G- (Cap-0), unmethyl-G-capped and uncapped RNA using mass spectrometry, intact mass of the relevant species was determined using LC/MS performed by external contractor Novatia LLC (Newtown, PA) or an in-house facility. The extent of RNA capping was assessed by the ratio of the mass intensity of the relevant RNase H-cleaved products.

FIGURE 14 shows the results of single-step transcription using currently commercially available reaction conditions. Comparing bar 1 and bar 2, the presence of VCE did not significantly affect the transcription output of T7 RNA polymerase but did not generate detectible m7GpppG- (Cap-0) transcript. A small fraction (5.4%) of

unmethylated-G capped RNA, an undesired capping reaction intermediate, was detected. Also, about 50% of the transcript contains a 5' diphosphate group, another capping reaction intermediate. As shown in bar 3, when Vaccinia cap 2'O methyltransferase was included in the reaction, transcription output was not significantly affected and no m7GpppGm- (Cap-1) transcript was detected. Approximately 50% of the transcript had a 5' diphosphate group and ~15% had the unmethyl-G capped intermediate. When Hi-T7 RNA polymerase was used instead of T7 RNA polymerase, results similar to T7 RNA polymerase reactions were observed – the presence of VCE did not impact transcription of Hi-T7 RNA polymerase significantly but did not generate detectible level of Cap-0 transcript. Addition of Vaccinia cap 2'O methyltransferase in the reaction did not produce any Cap-1 transcript. From these results, enzyme reagents and reaction conditions that are the current standard for *in vitro* mRNA synthesis (i.e. RNA synthesis followed by mRNA capping) do not support the *in vitro* production of capped mRNA in single reactions (i.e., single-step capped RNA synthesis).

B. Faustovirus RNA capping enzyme H3C2 efficiently caps transcripts in a single-step reaction *in vitro*

Since the reagents and conditions of Example 7A were ineffective to produce capped transcripts in a single-step reaction *in vitro*, new enzymes and reaction conditions were tested for their ability to synthesize single-step capped RNA synthesis *in vitro*. FIGURE 13 shows the results of single-step capped RNA synthesis using high concentration of H3C2 capping enzyme at 37°C or 45°C.

At 37°C, 0.5 µM of H3C2 capping enzyme generated 12% m7Gppp-capped transcript with as much as 24.1% unmethylated-G capped transcript as measured by capillary electrophoresis. At 45°C, on the other hand, as much as 61% of the transcripts had the m7Gppp- cap and only 1.4% of the transcripts had the unmethylated-G cap. At both temperatures, the transcription output was 50-60% of those without H3C2 capping enzyme. Hence, increasing H3C2 concentration and reaction temperature improved the yield of Cap-0 transcription production in single-step capped RNA synthesis using T7 RNA polymerase and H3C2 RNA capping enzyme.

The impact of further expanding the range of enzyme reagents and reaction temperature was tested by performing the single-step capped RNA synthesis at 45°C and 50°C Cap-0 using different combinations of T7 RNA polymerase, Hi-T7 RNA polymerase, VCE, H3C2 and Vaccinia cap 2'O methyltransferase. Specifically, single-step capped RNA synthesis reactions were carried out as described in Examples 7A using 0.5 µM VCE or H3C2 capping enzyme in the presence of absence of 5 U/µL Vaccinia mRNA cap 2'O methyltransferase in conjunction with 5U/µL T7 RNA polymerase or Hi-T7 RNA polymerase in their respective reaction buffering agents as indicated. Reactions were carried out at 45 or 50°C for 1 hour.

FIGURE 14 summarizes the results of this expanded survey. In bars 1-5, capped RNA synthesis reactions were carried out at 45°C using T7 RNA polymerase. In the presence of H3C2 capping enzyme, 84% of the transcript was m7Gppp- capped (Cap-0) and 8% was unmethylated-G capped (bar 2). In the presence of both H3C2 and cap 2'O methyltransferase, 87% of the transcript had the m7GpppGm-cap (Cap-1), 6% had the

unmethylated-G capped and no Cap-0 was detected (bar 3). When VCE was used, 76% of the transcript had the Cap-0 structure with 24% had the unmethylated-G cap structure (bar 4). In the presence of both VCE and cap 2'O methyltransferase, 55% of the transcript had the Cap-1 structure, 36% had the Cap-0 structure and 8% had the unmethylated-G cap structure. RNA yield was not significantly affected when H3C2 or VCE was present in reaction at 45°C. However, RNA yield decreased from 0.14 µg/µL to 0.09 µg/µL when cap 2'O methyltransferase and either of the capping enzymes were present. In capped RNA synthesis reactions using Hi-T7 RNA polymerase at 45°C (bars 6-10), a smaller fraction of transcript was capped compared to those using T7 RNA polymerase (bars 1-5). H3C2 capping enzyme and VCE generated 45% (bar 7) and 50% Cap-0 transcript (bar 9), respectively. H3C2 in conjunction with cap 2'O methyltransferase generated 44% Cap-1 transcript, 39% unmethylated-G capped transcript and no detectable Cap-0 transcript (bar 8). VCE in conjunction with cap 2'O methyltransferase generated 19% Cap-1 transcript, 22% Cap-0 transcript and 42% unmethylated-G capped transcript (bar 10). Interestingly, the transcription output of the reactions carried out at 45°C using Hi-T7 RNA polymerase was not significantly affected in the presence of capping enzyme or cap 2'O methyltransferase (bars 6-10). When the capped RNA synthesis reactions were carried out at 50°C using Hi-T7 RNA polymerase, H3C2 capping enzyme generated 80% Cap-0 transcript and 20% unmethylated-G capped transcript (bar 12). In the presence of both H3C2 and cap 2'O methyltransferase, 71% of the transcript contained Cap-1 structure, 2% contained Cap-0 structure and 26% contained the unmethylated-G cap structure (bar 13). When VCE was used, 55% of the transcript had the unmethylated-G capped structure and no Cap-0 transcript was detected. When both VCE and cap 2'O methyltransferase were present, 23% of the transcript had the Cap-0 structure and 31% had the unmethylated-G cap structure.

To summarize, this example provides single-step reaction conditions that produce *in vitro* Cap-0 RNA at up to 80% efficiency using H3C2 RNA capping enzyme in conjunction with T7 RNA polymerase or Hi-T7 RNA polymerase (Table 7). The example also describes single-step reaction conditions that can generate Cap-1 RNA at 70-80% efficiency using H3C2 RNA capping enzyme, Vaccinia mRNA cap 2'O methyltransferase in conjunction with T7 RNA polymerase or Hi-T7 RNA polymerase (Table 8).

Table 7

Cap 0 yield	RNAP	CE	Temp (°C)
≥80%	T7	H3C2	45°C
	Hi-T7	H3C2	50°C

Table 8

Cap 1 yield	RNAP	CE	2'O MTase	Temp(°C)
40-50%	Hi-T7	H3C2 or VCE	Vaccinia 2'O MTase	45°C
≥70%	T7	H3C2	Vaccinia 2'O MTase	45°C
	Hi-T7	H3C2	Vaccinia 2'O MTase	50°C

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Example 8: Single-step Cap-0 RNA synthesis using a H3C2:T7 RNA polymerase fusion protein

A fusion construct was prepared to produce protein composed of H3C2 RNA capping enzyme and T7 RNA polymerase linked by a putatively flexible linker sequence (SEQ ID NO:20). The fusion protein was encoded by DNA sequence (SEQ ID NO:21) under the control of T7 promoter or *tac* promoter, expressed in *E. coli* and purified using standard chromatographic techniques.

Single-step capped RNA synthesis reactions for the 1.7 kb *fluc* transcript were performed as indicated in Example 7 in 1x T7 RNA polymerase buffering agent supplemented with 19 mM MgCl₂ and 5 mM each of ATP, UTP, GTP, CTP and 0.1 mM SAM in the presence of 0.5 μM H3C2:T7 RNA polymerase fusion. Reactions containing 5 U/μL T7 RNA polymerase with or without 0.5 μM H3C2 capping enzyme were done for comparison. The reactions were carried out at 37 or 45°C for 1 hour. The RNA yield and fraction of capped and uncapped transcription were measured and analyzed as indicated in Example 7.

Results are shown in FIGURE 15. When the reactions were performed at 37°C, the H3C2:T7 fusion protein generated 75% m7Gppp-capped (Cap-0) transcript and 20% unmethylated-G capped transcript (bar 1). Under the same conditions, separate enzymes H3C2 and T7 RNA polymerase only generated 12% Cap-0 transcript and 27% unmethylated-G capped transcript (bar 2). The transcription output decreased from 0.45 μg/μL when only T7 RNA polymerase was present (bar 3) to 0.39 μg/μL when H3C2 was added and further to 0.19 μg/μL when H3C2:T7 fusion was used for transcription. At 45°C, the H3C2:T7 RNA polymerase fusion generated 95% Cap-0 transcript and 5% unmethylated-G capped transcript (bar 4), compared to 60% Cap-0 and 6% unmethyl-G capped transcript when individual enzymes were used (bar 5). Similar to reactions performed at 37°C, the transcription output decreased from 0.31 μg/μL when only T7 RNA polymerase was present (bar 6) to 0.18 μg/μL when H3C2 was added or to 0.12 μg/μL when H3C2:T7 fusion was used for transcription.

Thus, under the reaction conditions described, this example demonstrates efficient (*e.g.*, as high as 98%), single-step *in vitro* Cap-0 RNA synthesis using an H3C2:T7 RNA polymerase fusion protein.

Example 9: RNA capping enzymes efficiently caps *in vitro* transcripts containing pseudo-uridine

A ~1800 nt RNA encoding the cypridina luciferase protein with a 120 nt poly(A) tail (*cluc/A120*) was synthesized with *in vitro* transcription using T7 RNA polymerase (New England Biolabs Inc.) in the presence of pseudouridine triphosphate (Trilink Biotechnologies) or unmodified uridine triphosphate (New England Biolabs Inc.). 500 nM of unmodified or pseudouridine *cluc/A120* transcript was incubated with 100 nM of H3C2 or VCE

in the presence of 0.5 mM GTP and 0.1 mM SAM in 10 mL of 1x RNA capping buffering agent (50 mM Tris-HCl, 5 mM KCl, 1 mM MgCl₂, 1 mM DTT, pH 8) at 37°C for 30 min. A targeting oligo (TO) designed to direct RNase H to cleave out a 5' fragment was then added to each capping reaction to achieve a final TO concentration of 2.5 μM. Each reaction was then heated at 80°C for 30 s to anneal the TO to the transcript and inactivate the capping enzymes and cooled slowly to room temperature. Thermostable RNase H (New England Biolabs) was added to each reaction at a final concentration of 0.5 U/mL and incubated at 37°C for 1h. Reaction products were then purified and analyzed by intact LC/MS as described in Example 7. As shown in FIGURE 16, VCE and H3C2 m⁷Gppp-capped 68.4% or 100% of the *cluc/A120* transcript containing pseudouridine, respectively. Notably, both capping enzymes cap a larger fraction of the pseudouridine-containing *cluc/A120* transcript than the uridine counterpart.

Example 10: Scaled-up Cap-1 capping and functional assay

A total of 250 pmol (145 μg) of ~1800 nt *cluc/A120* *in vitro* transcript was capped in a 500 μL reaction containing 1x RNA capping buffering agent (50 mM Tris-HCl, pH 8.0, 5 mM KCl, 1 mM MgCl₂, 1 mM DTT), 0.5 mM GTP, 0.1 mM SAM, 10 U/μL of Vaccinia cap 2'O methyltransferase (NEB M0366) and 500 nM of H3C2 at 45°C for 1 h. The efficiency of Cap-1 formation was assessed by the RNase H/intact LC/MS as described in Example 7. The RNA was purified using acidic phenol and chloroform, followed by ethanol precipitation, and rehydrated in nuclease-free water. The RNA concentration was estimated by Qubit RNA (Broad Range) kit (ThermoFisher). The translation efficiency of the capped *cluc/A120* transcript was measured as relative luciferase activity from HEK293 cells transfected with the capped *cluc/A120* transcript in relation to uncapped *cluc/A120* transcript. HEK293 cells were transfected with 250 ng of Purified *cluc/A120* transcript \ using Lipofectamine MessengerMax transfection reagent (ThermoFisher). Luciferase activity was assayed four hours post-transfection on 10 μL of culture medium supernatant using the BioLux® Cypridina Luciferase Assay Kit (New England Biolabs Inc. Ipswich) . Luminescence was measured using Centro LB960 microplate luminometer from Berthold technologies. As shown in FIGURE 17, the scale-up capping reaction achieved 47.7% and 54.8% Cap-1 formation on *cluc/A120* containing rU and pseudouridine, respectively, consistent to the results in Example 5 and FIGURE 11. The luciferase assay results in triplicate showed that the translation efficiency from the purified capped transcripts containing rU and pseudouridine exhibited high luciferase activity (FIGURE 18), indicating that the RNAs capped with H3C2 at 45°C were functionally active. It should be noted that modifications to reaction conditions such as reaction volume, concentration of enzymes and reaction components, reaction time and reaction temperature may improve the Cap-1 formation efficiency of a larger quantity of RNA and the translation efficiency of the resultant Cap-1 RNA.

Example 11: Single-vessel Capped RNA synthesis with multiple temperature steps

Single-vessel capped RNA synthesis reactions for the 1.7 kb *fluc* transcript were performed as indicated in Example 7, namely, 5 U/ul of T7 RNA polymerase (New England Biolabs; Cat. No. M0251) was incubated with or without 500 nM of Vaccinia capping enzyme (New England Biolabs; Cat. No. M2080) or H3C2 capping enzyme for 37°C for 30 min, followed by another 30-min incubation at 28°C, 32°C, 37°C, 45°C, or 50°C. The reactions contained 1x T7 RNA polymerase buffering agent (40 mM Tris-HCl, pH 7.9, 10 mM NaCl, 1 mM DTT, 2 mM spermidine) and were supplemented with 19 mM MgCl₂ and 5 mM each of ATP, UTP, GTP, CTP and 0.5 mM SAM. The transcription output was analyzed by DNase I/Qubit quantitation as described in Example 7. The capping efficiency of the reactions were evaluated by RNase H cleavage followed by Klenow fill-in with FAM-dCTP and urea-PAGE as indicated in Example 7. Selected reactions were further analyzed by intact mass spectrometer. As shown in FIGURE 19, for VCE, the m⁷Gppp-forming efficiency peaked at second reaction temperature of 45°C, whereas for H3C2, the m⁷Gppp-forming efficiency peaked at second reaction temperature of 50°C. T7 RNAP transcription output, on the other hand, peaked at 45°C when paired with either VCE or H3C2, reflecting the low transcription activity at the lower and upper end of the tested temperatures (28°C and 50°C, respectively). Hence, tandem temperature steps of 37°C for 30 min followed by 45°C for 30 min yielded the optimal balance of transcription output and capping efficiency in this example. Modifications to reaction conditions such as the reaction time, reaction temperature and the number of temperature steps may further improve the transcription yield and fraction of m⁷G-capped transcript.

CLAIMS

What is claimed is:

1. A method for capping RNA *in vitro*, comprising:
contacting:
 - (i) an RNA sample comprising an uncapped target RNA;
 - (ii) an RNA capping enzyme comprising an amino acid sequence that is at least 90% identical to SEQ ID NO:1, 7, or 20;
 - (iii) guanosine triphosphate (GTP) or modified GTP
 - (iv) a buffering agent; and
 - (v) a methyl group donor,at a temperature of 40°C-60°C to form a capped target RNA.
2. A method for efficiently capping RNA *in vitro*, comprising:
contacting:
 - (i) an RNA sample comprising an uncapped target RNA;
 - (ii) a single-chain RNA capping enzyme that has RNA triphosphatase (TPase), guanylyltransferase (GTase) and guanine-N7 methyltransferase (N7 MTase) activities;
 - (iii) guanosine triphosphate (GTP) or modified GTP
 - (iv) a buffering agent; and
 - (v) a methyl group donor,at a temperature of 37°C-60°C, to form a capped target RNA.
3. The method of claim 1 or 2, wherein the uncapped RNA target is at least 200 nt in length.
4. The method of claim 1 or 2 or 3, wherein contacting further comprises increasing or decreasing the temperature to a second temperature of 37°C-60°C, wherein the second temperature differs from the first temperature.
5. The method of claim 2, wherein the single-chain RNA capping enzyme comprises:
 - (a) amino acid sequence at least 90% identical to (x) SEQ ID NO:2, (y) SEQ ID NO:3, and/or (z) SEQ ID NO:4; or
 - (b) an amino acid sequence at least 90% identical to (x) SEQ ID NO:5 and/or (y) SEQ ID NO:6.
6. The method of claim 2, wherein the single-chain RNA capping enzyme comprises an amino acid sequence at least 90% identical to (a) SEQ ID NO:7, (b) SEQ ID NO:8, (c) SEQ ID NO:9, (d) SEQ ID NO:10, (e) SEQ ID NO:11 or (f) SEQ ID NO:12.

7. The method of any prior claim, wherein (i), (ii), (iii), and (iv) are RNase-free and contacting optionally further comprises contacting (v) one or more RNase inhibitors.
8. The method of any prior claim, further comprising synthesizing the uncapped RNA using solid-phase oligonucleotide synthesis chemistry.
9. The method of any prior claim, further comprising synthesizing the uncapped RNA by contacting a DNA template encoding the uncapped RNA and a polymerase to produce the uncapped RNA.
10. The method of any prior claim, wherein the methyl group donor is S-adenosyl methionine and contacting further comprises contacting (vi) a cap 2'O methyltransferase enzyme.
11. The method according to any prior claim, wherein the uncapped target RNA comprises one or more pseudouridines.
12. The method of any prior claim, wherein contacting further comprises contacting (i), (ii), (iii), (iv) and optionally (v) in a single location.
13. A composition comprising:
 - (i) an uncapped target RNA;
 - (ii) a single-chain RNA capping enzyme that has RNA triphosphatase (TPase), guanylyltransferase (GTase) and guanine-N7 methyltransferase (N7 MTase) activities;
 - (iii) guanosine triphosphate (GTP);
 - (iv) a buffering agent; and
 - (v) a methyl group donor.
14. The composition of claim 13, wherein the composition has a temperature in the range of 37°C-60°C.
15. The composition of claim 12, wherein the composition is RNase-free and optionally comprises (v) one or more RNase inhibitors.
16. The composition of any of claims 13-15, wherein the single-chain RNA capping enzyme comprises:
 - (a) amino acid sequences that are at least 90% identical to (x) SEQ ID NO:2, (y) SEQ ID NO:3, and/or (z) SEQ ID NO:4; or
 - (b) an amino acid sequence at least 90% identical to (x) SEQ ID NO:5 and/or (y) SEQ ID NO:6.

17. The composition of any of claims 13-16, wherein the single-chain RNA capping enzyme comprises an amino acid sequence at least 90% identical to (a) SEQ ID NO:7, (b) SEQ ID NO:8, (c) SEQ ID NO:9, (d) SEQ ID NO:10), (e) SEQ ID NO:11 or (f) SEQ ID NO:12.
18. The composition of any of claims 13-17, wherein the composition further comprises a DNA template, a bacteriophage polymerase and ribonucleotide triphosphates, for transcribing the RNA.
19. The composition of any of claims 13-18, wherein the composition optionally comprises (vi) S-adenosyl methionine (SAM) and (vii) a cap 2'O methyltransferase enzyme.
20. The composition of any of claims 13-19, wherein the uncapped target RNA comprises one or more pseudouridines.
21. A kit comprising:
 - a single-chain RNA capping enzyme that has RNA triphosphatase (TPase), guanylyltransferase (GTase) and guanine-N7 methyltransferase (N7 MTase) activities, wherein the enzyme is in a storage buffering agent; and
 - a reaction buffering agent.
22. The kit of claim 20, wherein the kit further comprises a bacteriophage polymerase and ribonucleotides, for transcribing a template polynucleotide encoding a target RNA.
23. The kit of claim 21 or 22, wherein the kit further comprises S-adenosyl methionine (SAM), . cap 2'O methyltransferase enzyme (2'OMTase), or both SAM and 2'OMTase.
24. The kit of any of claims 20-23, wherein the single-chain RNA capping enzyme comprises:
 - (a) amino acid sequences that are at least 90% identical to (x) SEQ ID NO:2, (y) SEQ ID NO:3, and/or (z) SEQ ID NO:4 or
 - (b) an amino acid sequence that is at least 90% identical to (x) SEQ ID NO:5 and/or (y) SEQ ID NO:6.
25. The kit of any of claims 20-23, wherein the single-chain RNA capping enzyme comprises an amino acid sequence that is at least 90% identical to (a) SEQ ID NO:7, (b) SEQ ID NO:8, (c) SEQ ID NO:9, (d) SEQ ID NO:10), (e) SEQ ID NO:11 or (f) SEQ ID NO:12.
26. An RNA capping enzyme comprising an amino acid sequence that is at least 90% identical to SEQ ID NO:20.

27. An RNA capping enzyme fusion comprising (a) an amino acid sequence that is at least 90% identical to SEQ ID NO:7 and (b) an amino acid sequence that is at least 90% identical to positions 1419 to 1587 of SEQ ID NO:20.

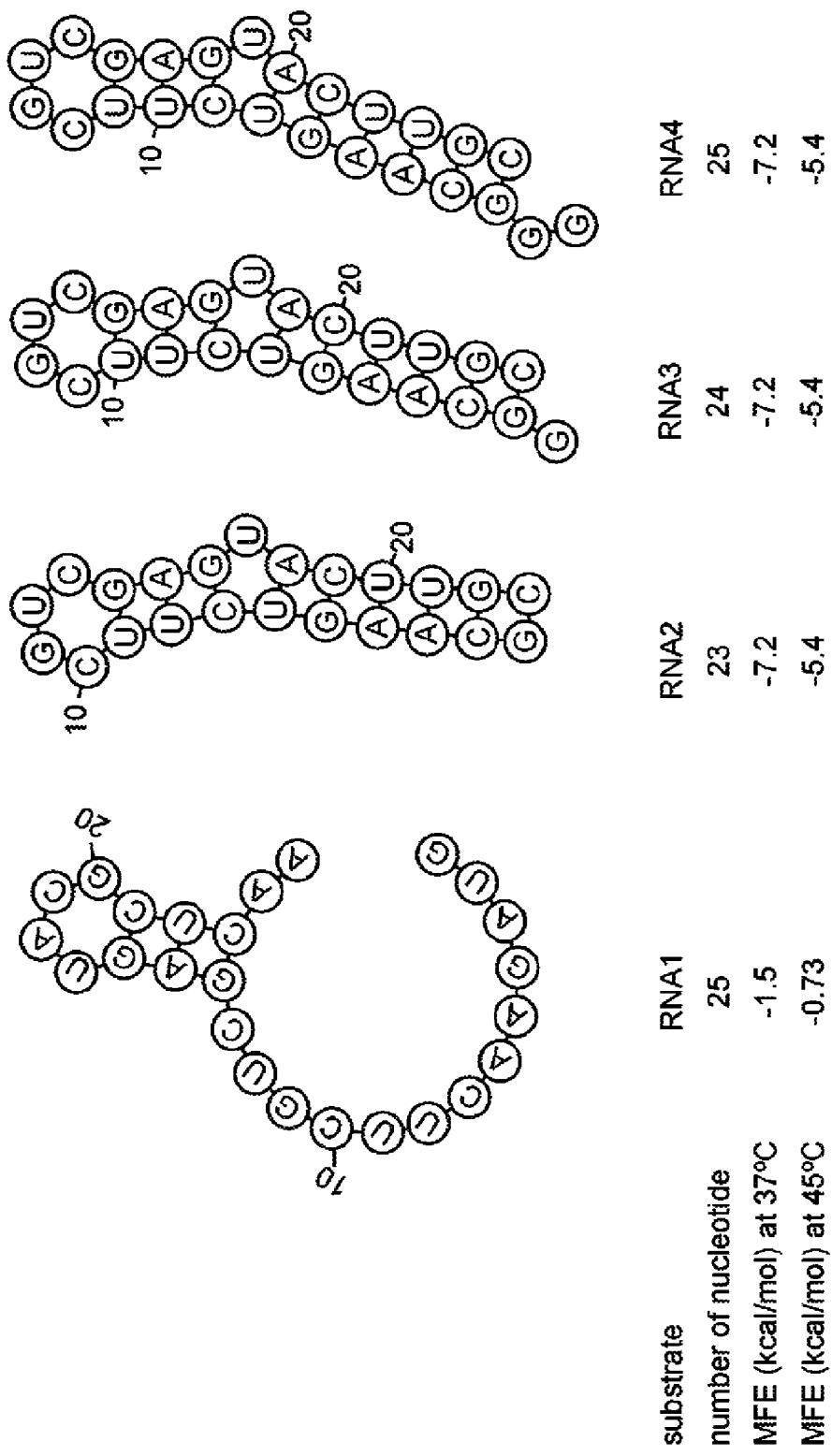


FIG. 1

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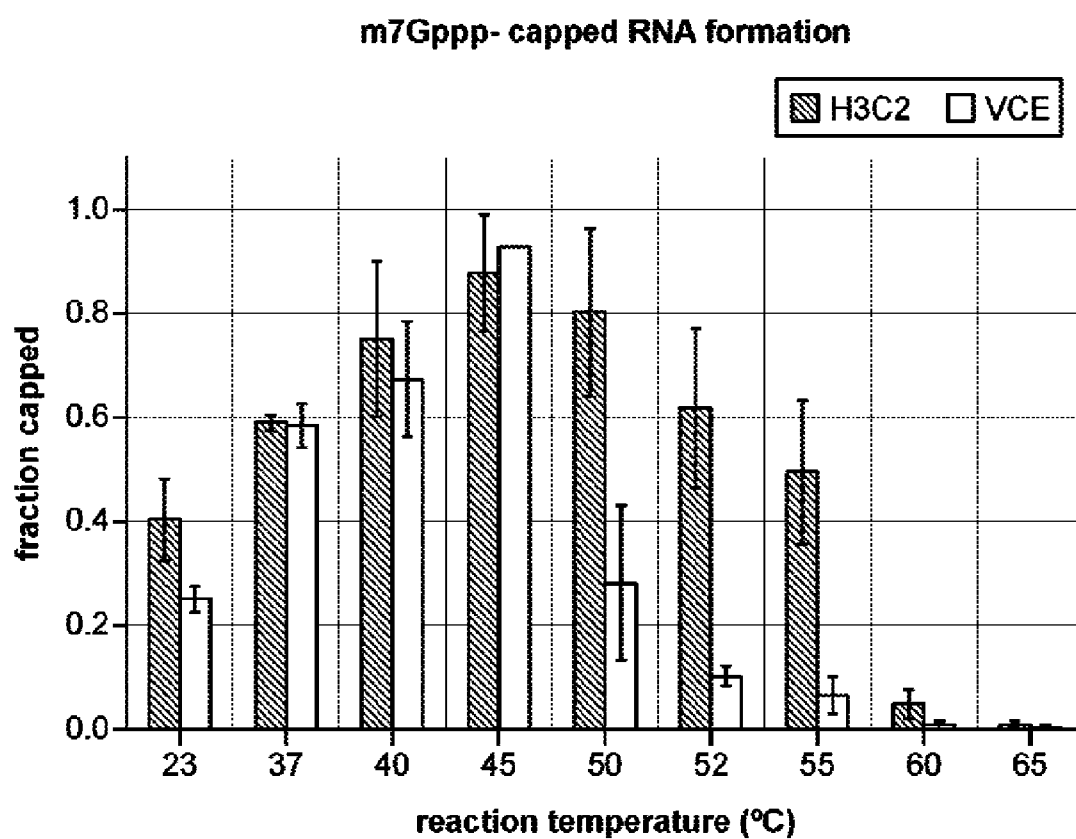


FIG. 2

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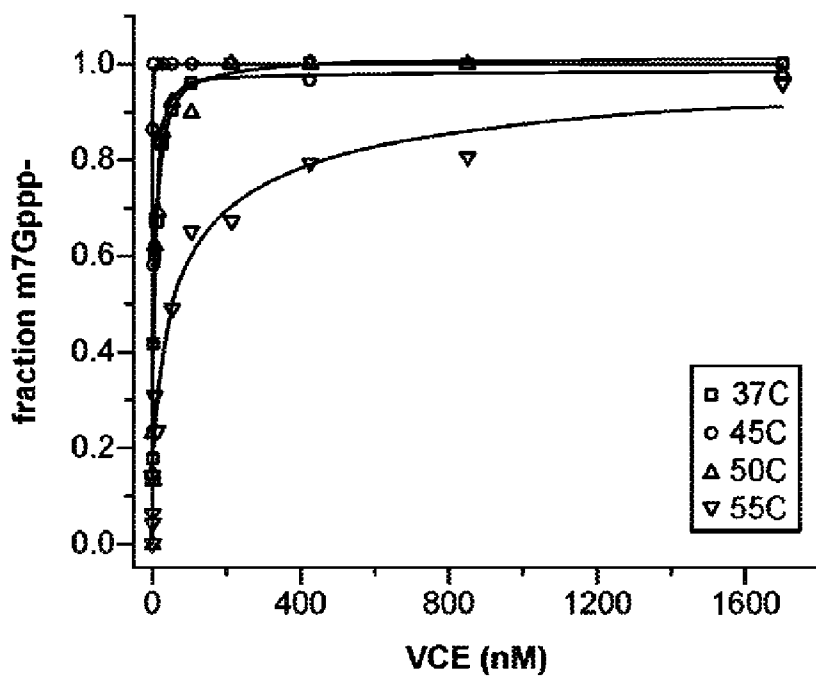


FIG. 3A

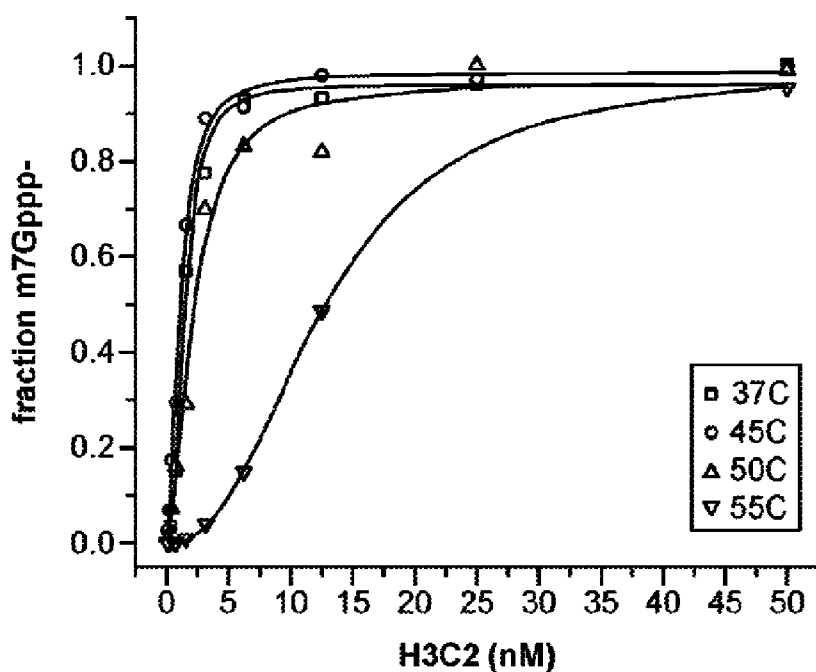


FIG. 3B

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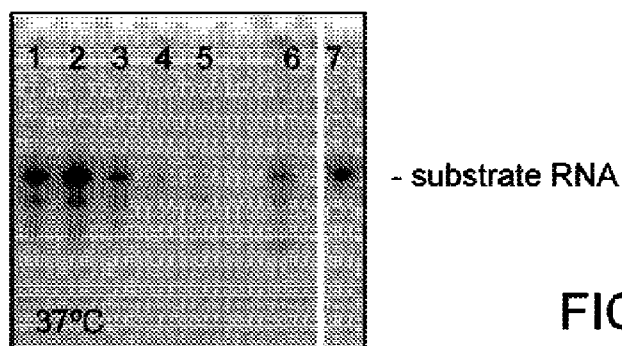


FIG. 4A

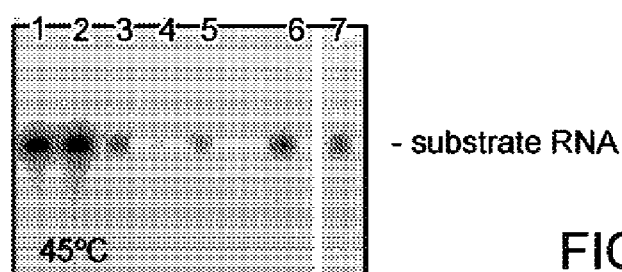


FIG. 4B

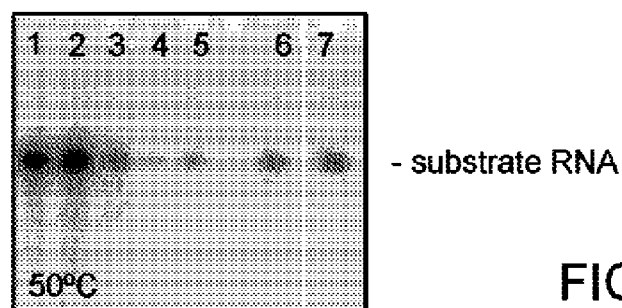


FIG. 4C

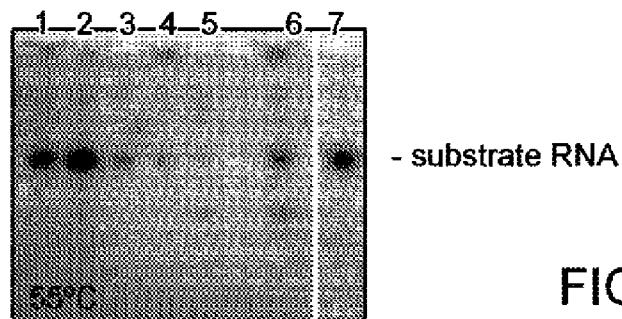


FIG. 4D

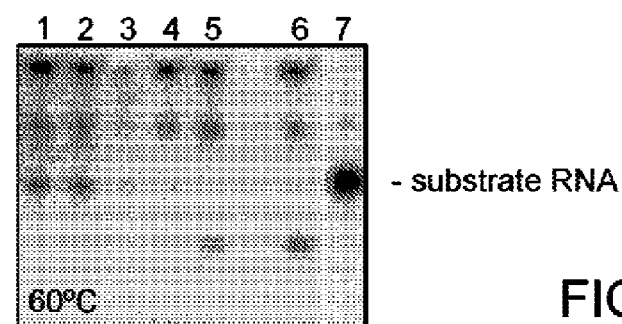


FIG. 4E

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FIG. 5 (Cont. 1)

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AMN83561 500 510 520 530 540 550 560
|....|....|....|....|....|....|....|....|....|
 468 ILFTDINMFGSRVPIQFQPAINPLAYVCYLPEDVN-----VNDGDIVEMRAVDGYDTIPKWELVRSRND
 AIB52055 484 LLFTDVNLFGNKIPIQFMPAINPLAYICYLPSSGT-----VNDGDIVEMRAVDGFGDGIPTWELVTRPD
 SME65026 484 LLFTDVNLFGNKIPIQFMPAINPLAYICYLPSSGT-----VNDGDIVEMRAVDGFGDGIPTWELVTRPD
 SMH63629 484 MLFTDINLFGRVPIQFMPAINPLAYICYLPEAATCANGDAINDGDIVEMRAVDGFDTVPKWEPIRVRSD

570 580 590 600 610 620 630
|....|....|....|....|....|....|....|....|....|
 532 RKNEPGFYGNKYKIASDIYLNID VFHEEDLYKYNPGYFEKNKSDIY VAPNKYRRYLLIKSLFGRYLRDAK
 AIB52055 548 RKDERGFYGNKYKIASDIYLNID VENEDDLWKYNPGYFEKNKSDIY IAPNKYRRYLLIKSLFNKYIKNAK
 SME65026 548 RKDERGFYGNKYKIASDIYLNID VENEDDLWKYNPGYFEKNKSDIY IAPNKYRRYLLIKSLFNKYIKNAK
 SMH63629 554 RKDERGFYGNKYKIASDIYLNID IFQFEDLYKYNPGYFEKNKSDIY VAPNKYRRYLLIKSLFNKYIKNAK
 Fausto_CR_03 1 -----GFYGNKYKIASDIYLNID VENEDDLWKYNPGYFEKNKSDIY IAPNKYRRYLLIKSLFNKYIKNAK

640 650 660 670 680 690 700
|....|....|....|....|....|....|....|....|....|
 602 WVTDAAAGRGADLHLYKAECEVENLLAIDIDPTAISSELVRRRNEITGYNKSHRGGR--NMHSHRGQSH---
 AIB52055 618 WVTDAAAGRGADLHLYKQECVENLLAIDIDPTAISSELVRRRNEITGWQQRGGG----NTRHNARHN---
 SME65026 618 WVTDAAAGRGADLHLYKQECVENLLAIDIDPTAISSELVRRRNEITGWQQRGGGMMHNSRHNTRHN---
 SMH63629 624 WVTDAAAGRGADLHLYKAECEVENLLAIDIDPTAISSELVRRRNEITGYNRGHRGHRGGSMRAHMGASHHGA
 Fausto_CR_03 66 WVTDAAAGRGADLHLYKAECEVENLLAIDIDPTAISSELVRRRNEITG-----

FIG. 5 (Cont. 2)

	710	720	730	740	750	760	770
AMN83561	666	666	666	666	666	666	666
AIB52055	681	681	681	681	681	681	681
SME65026	685	685	685	685	685	685	685
SMH63629	694	694	694	694	694	694	694
Fausto CR_05	735	735	735	735	735	735	735
	751	751	751	751	751	751	751
	755	755	755	755	755	755	755
	764	764	764	764	764	764	764
	804	804	804	804	804	804	804
	821	821	821	821	821	821	821
	825	825	825	825	825	825	825
	832	832	832	832	832	832	832
	840	840	840	840	840	840	840

FIG. 5 (Cont. 3)

	290	300	310	320	330	340	350
							
280	INFIPNRYAVTDKADGERYFLFSINSGIYLLSINLTVKKINIPVLEKRYQNMLIDGEYIKTTGHDLFMVF						
275	VKEFVPNRYAITDKADGERYFLFSTPNGVYLLSINLTVKKVNIFVLQKDFONMLDGEILIDIDGKELEFMVF						

FIG. 6

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AAV50651 YP_007354410	350	DVIFAEGTDYRYDNTYSLPKRIIIINNIIDKCFGNLIIPFNDYTDKHNNELEDSIKTYYSKSELSNYWKNEK	360	370	380	390	400	410	420
	345	DVYHNGIDYRYDNTYTLTHRIIIINDIIDKAFNNLIIPFTDYTDKYNNELEDKIKEFYSNEIKTYWKNEFS							
AAV50651 YP_007354410	420	NRLNKSTDLFTTRKLYLVPGIDSSSEIFMYADMIWKLIVVNELTYPYQLDGIITYTPINSPYLIRGGIDAYD	430	440	450	460	470	480	490
	415	KKLKNYSGLETSRKLYFVPGIDSSSEVEMYADLVWKLVCVDQLTPYKLDGIITYTPIASPYMIKTSANELE							
AAV50651 YP_007354410	490	TIPMEYKWKPPSONSIDFYIRFKDVSADAVYYDNSVERAEGKPYKICLLIYVGLNKQGQEIPIQFKVNG	500	510	520	530	540	550	560
	485	SVMEYKWKPPSONSIDFYVKFDKARGNEAIYYDNAVRGGRPYKVCGLFVGLNKSGGEEKPIAFKVAG							
AAV50651 YP_007354410 moumou_CR_03	560	VEQTANIYTKDGEATDINGNAINDNTVVEFEEDTLKIDMDDSYKWIPITRTRYDKTESVQKYHKHYGNNLQ	570	580	590	600	610	620	630
	555	VEQKAFIYLTNDEALDLSGNVINDNTVVEFEEDNFKIDMDDPYKWIPITRTRYDKTESVQKYRKHYGNNLH							
	1	INDNTVVEFEEDNFKIDMDDPYKWIPITRTRYDKTESVQKYHKHYGNNLH							
AAV50651 YP_007354410 moumou_CR_03	630	TANRIWKTIINPTIEDITISLGDPTTENKEITLISDFRDKYNKQALITYYQKNTSNAAGMRAFNWIKSN	640	650	660	670	680	690	700
	625	TANRIWETITINPVTEBIIAALGNASTFEKEMSKLVKMNES-YNKQSFYYQKNTSNAIGMRAFNNEIKSN							
	50	TANRIWKTIINPTIEDILI							
	1	YYQKNTSNAAGMRAFNNEIKSN							

FIG. 6 (Cont. 1)

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AAV50651	700	710	720	730	740	750	760	770
YP 007354410		MTase						
mod mou CR 04								
	23	MITTYQDGSKVIDIGCGRGGLIKFTINAGVEFYVGIQIDNGLYVINDSANNRYKNLKKTIQNIPEPMTE						
		MITTYQKOKDSVLDIGCGRGGLIKFTINAGVEFYVGIQIDNGLYVINDSANNRYKNLKKTIQNIPEPMTE						
		MITTYQKOKGKVIDIGCGRGGLIKFTINAGVEFYVGIQIDNGLYVINDSANNRYKNLKKTIQNIPEPMTE						
		INADARGLEITEAOKILPGMPDNFKSLINKYLGN-KYDTINCOFTIHYLSDELWNNECKNINNOLK						
AAV50651	770	780	790	800	810	820	830	840
YP 007354410		MTase						
mod mou CR 04								
	93	INADARGLEITEAOKILPGMPDNFKSLINKYLGN-KYDTINCOFTIHYLSDELWNNECKNINNOLK						
		INADARGLEITEAOKILPGMPDNFKSLINKYLGN-KYDTINCOFTIHYLSDELWNNECKNINNOLK						
		INADARGLEITEAOKILPGMPDNFKSLINKYLGN-KYDTINCOFTIHYLSDELWNNECKNINNOLK						
		INADARGLEITEAOKILPGMPDNFKSLINKYLGN-KYDTINCOFTIHYLSDELWNNECKNINNOLK						
AAV50651	839	850	860	870	880	890	900	910
YP 007354410		MTase						
	834	DNGYLLITSDGNLIHNKLGKQKLSSTYTDNRGNKNIFFEINKIYSDTDKVGLGMAIDLINSLISNPGT						
		DNGYLLITCFDGLIYDKLGKQKLSSTYTDNRGNKNIFFEINKIYSDTDKVGLGMAIDLINSLISNPGT						
		DNGYLLITCFDGLIYDKLGKQKLSSTYTDNRGNKNIFFEINKIYSDTDKVGLGMAIDLINSLISNPGT						
		DNGYLLITCFDGLIYDKLGKQKLSSTYTDNRGNKNIFFEINKIYSDTDKVGLGMAIDLINSLISNPGT						
AAV50651	909	920	930	940	950	960	970	980
YP 007354410		MTase						
	904	YIREYLVFPEFLEKSLKECCGLELVEVDLQYNIENFYKNIYKFTYNEGYMTDVSSKKHSEIREFYLSLEG						
		YQREYLVFPDFLQKSLKQCGLELVEVDLQYNIENFYKNIYKFTYNEGYMTDVSSKKHSEIREFYLSLEG						
		YQREYLVFPDFLQKSLKQCGLELVEVDLQYNIENFYKNIYKFTYNEGYMTDVSSKKHSEIREFYLSLEG						
		YQREYLVFPDFLQKSLKQCGLELVEVDLQYNIENFYKNIYKFTYNEGYMTDVSSKKHSEIREFYLSLEG						
AAV50651	979	990	1000	1010	1020	1030	1040	1050
YP 007354410		MTase						
	974	NANNDIEIDIAASAFKIAMLNRYVYVFRKSTINITEPSRIVNEIINNRIIDLGKFTIMPYFRTNNMFIDLDNV						
		KSSSVIESDIAASAFKIAMLNRYVYVFRKSTINITEPSRIVNEIINNRIIDLGKFTIMPYFRTNNMFIDLDNV						
		KSSSVIESDIAASAFKIAMLNRYVYVFRKSTINITEPSRIVNEIINNRIIDLGKFTIMPYFRTNNMFIDLDNV						
		KSSSVIESDIAASAFKIAMLNRYVYVFRKSTINITEPSRIVNEIINNRIIDLGKFTIMPYFRTNNMFIDLDNV						
AAV50651	1049	1060	1070	1080	1090	1100	1110	1120
YP 007354410		MTase						
	1044	DTDINRVYRNIRNKYRTTRPHVYLKHNINENRLEDIYLSNNKIDFSKIKNGSDPKVLLIYKSPDKQFYF						
		NNDVNKIYHFIKKKYSPIKPSVYIVRHNIIIDNEMDGTTFSRNKLEFIKIKNGTDPKVLLIYKSPDKQFYF						
		NNDVNKIYHFIKKKYSPIKPSVYIVRHNIIIDNEMDGTTFSRNKLEFIKIKNGTDPKVLLIYKSPDKQFYF						
		NNDVNKIYHFIKKKYSPIKPSVYIVRHNIIIDNEMDGTTFSRNKLEFIKIKNGTDPKVLLIYKSPDKQFYF						
AAV50651	1119	1130	1140	1150	1160	1170		
YP 007354410		MTase						
	1114	IYYQNYQSMFPDLD---						
		IYYQNYQSMFPDLD---						
		IYYQNYQSMFPDLD---						
		IYYQNYQSMFPDLD---						

FIG. 6 (Cont. 2)

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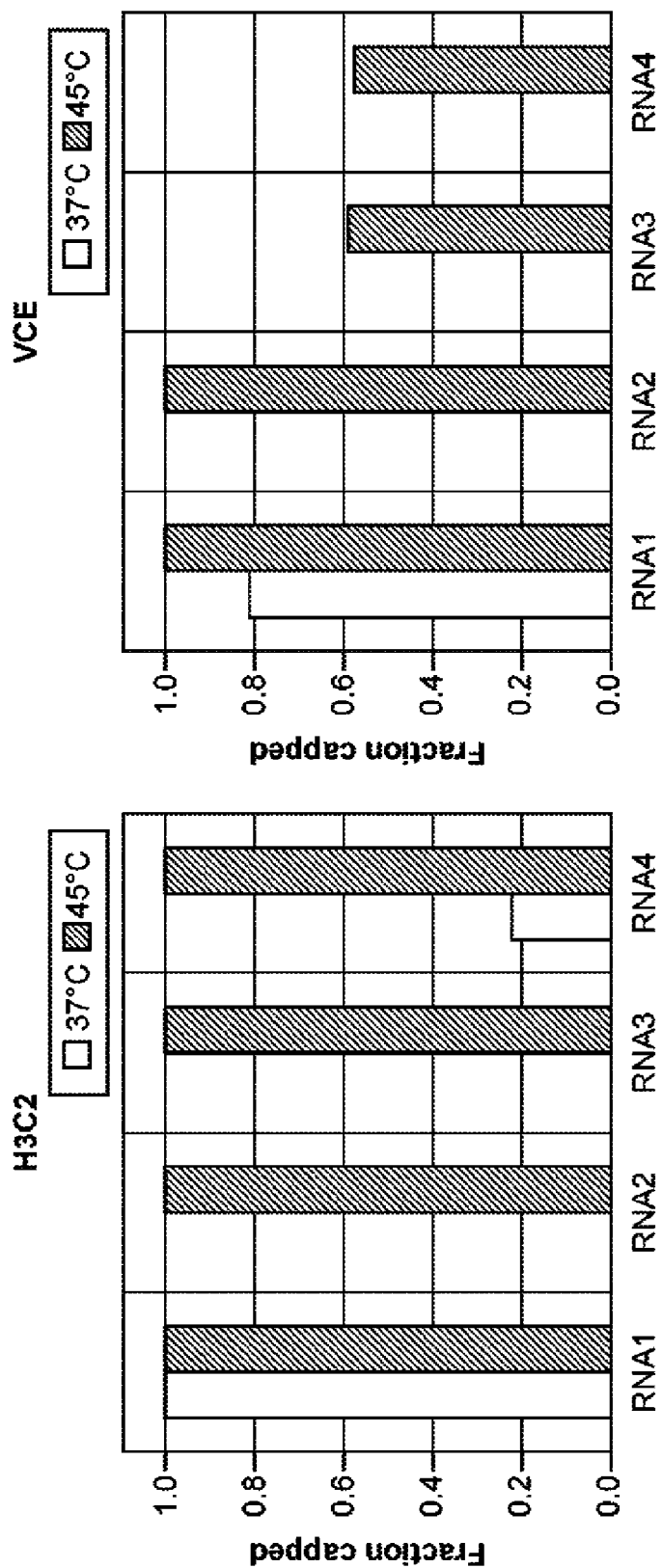


FIG. 7

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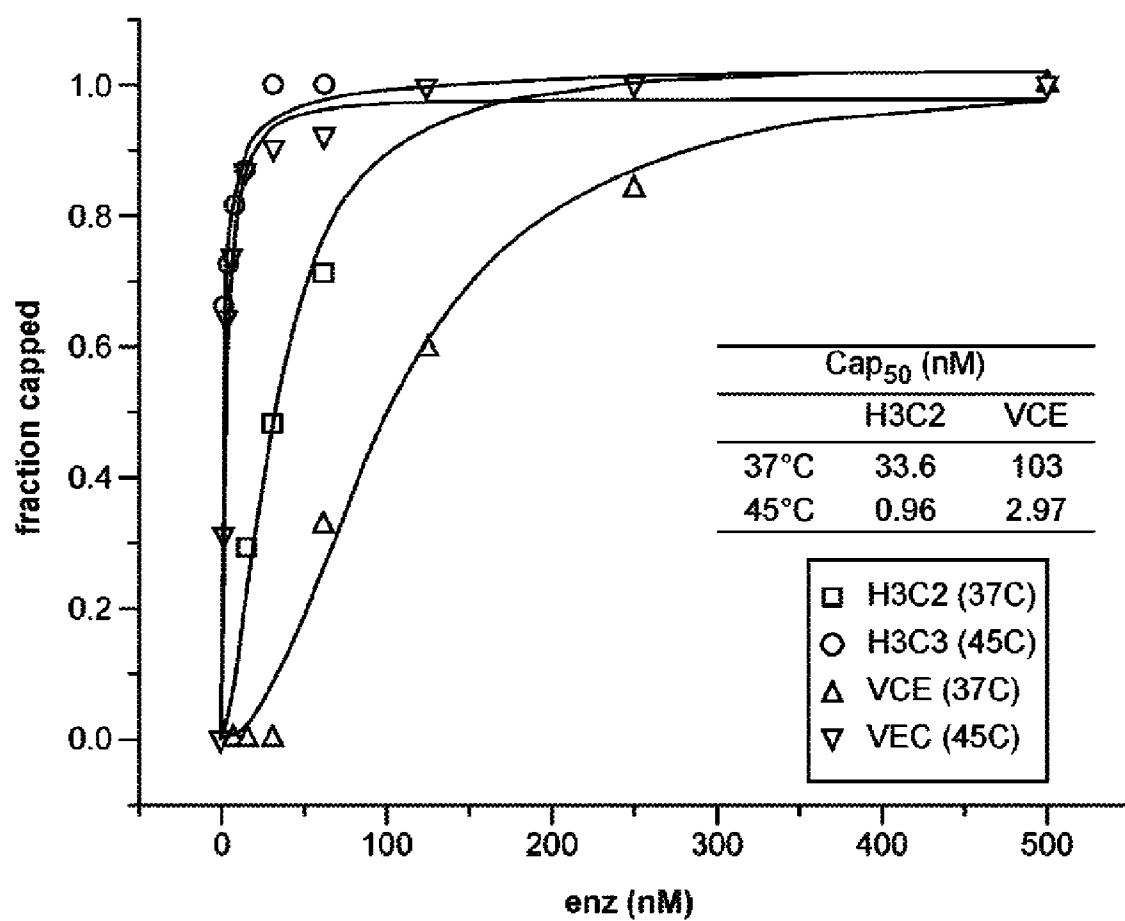


FIG. 8

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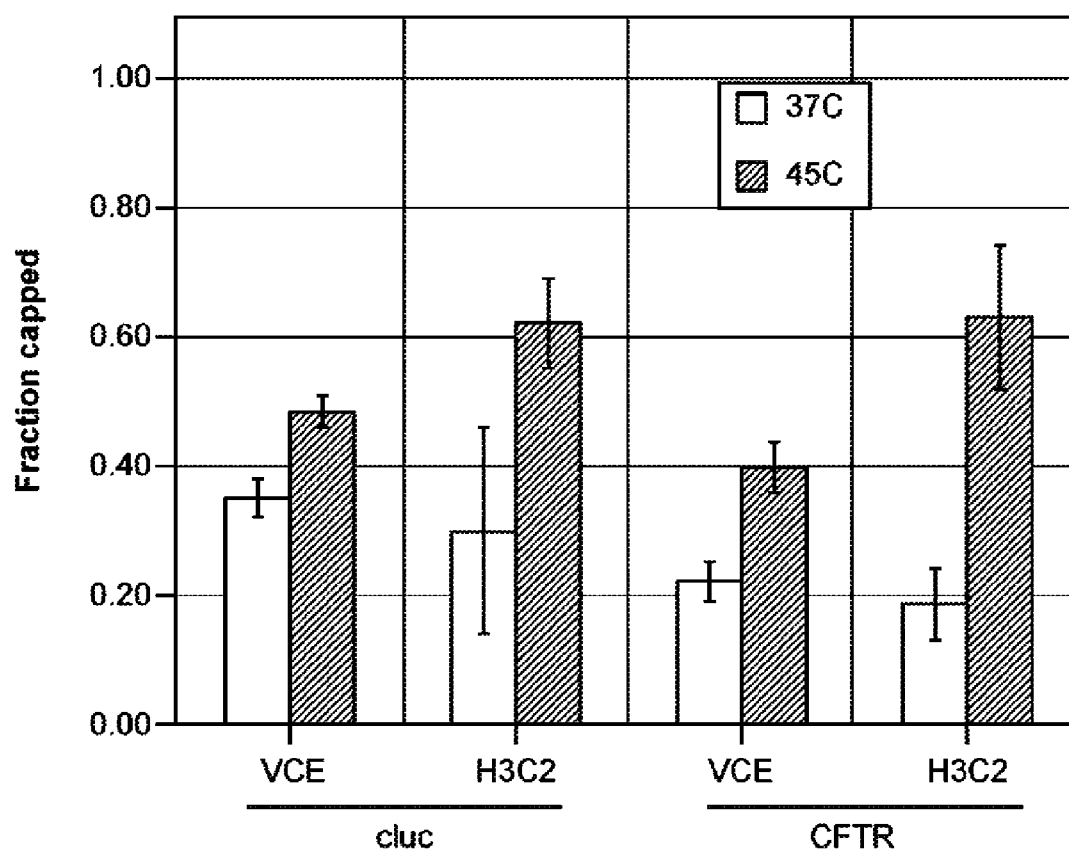


FIG. 9

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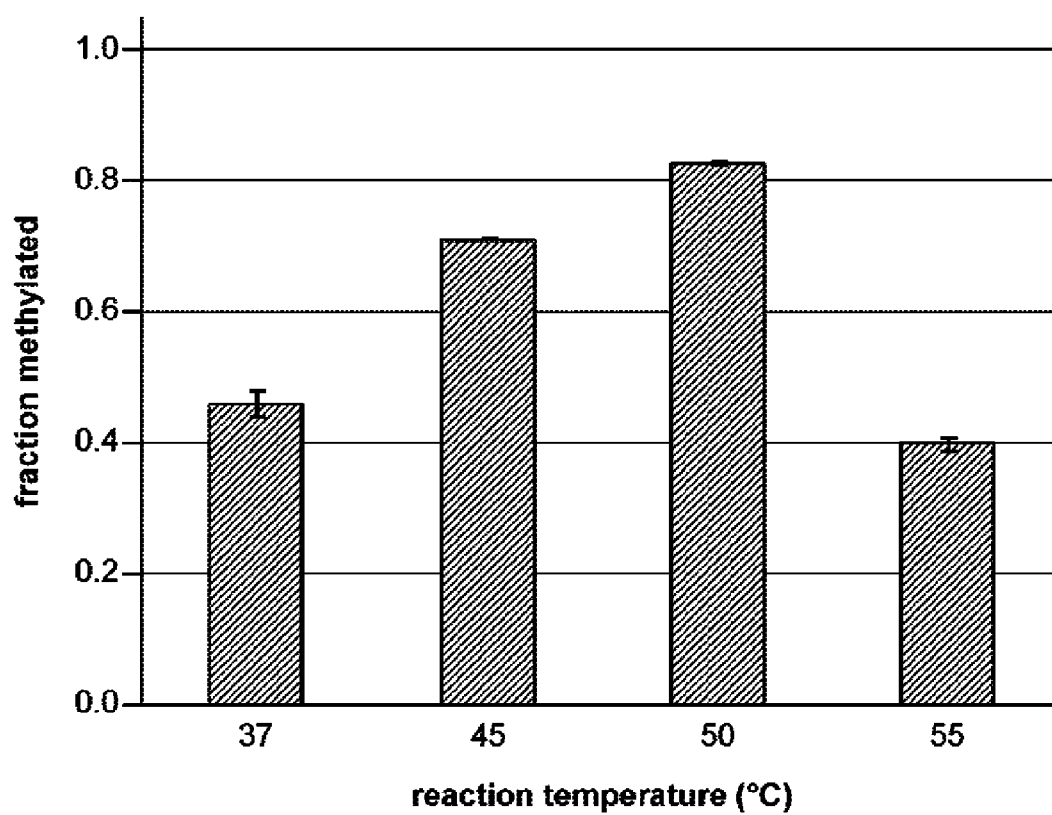


FIG. 10

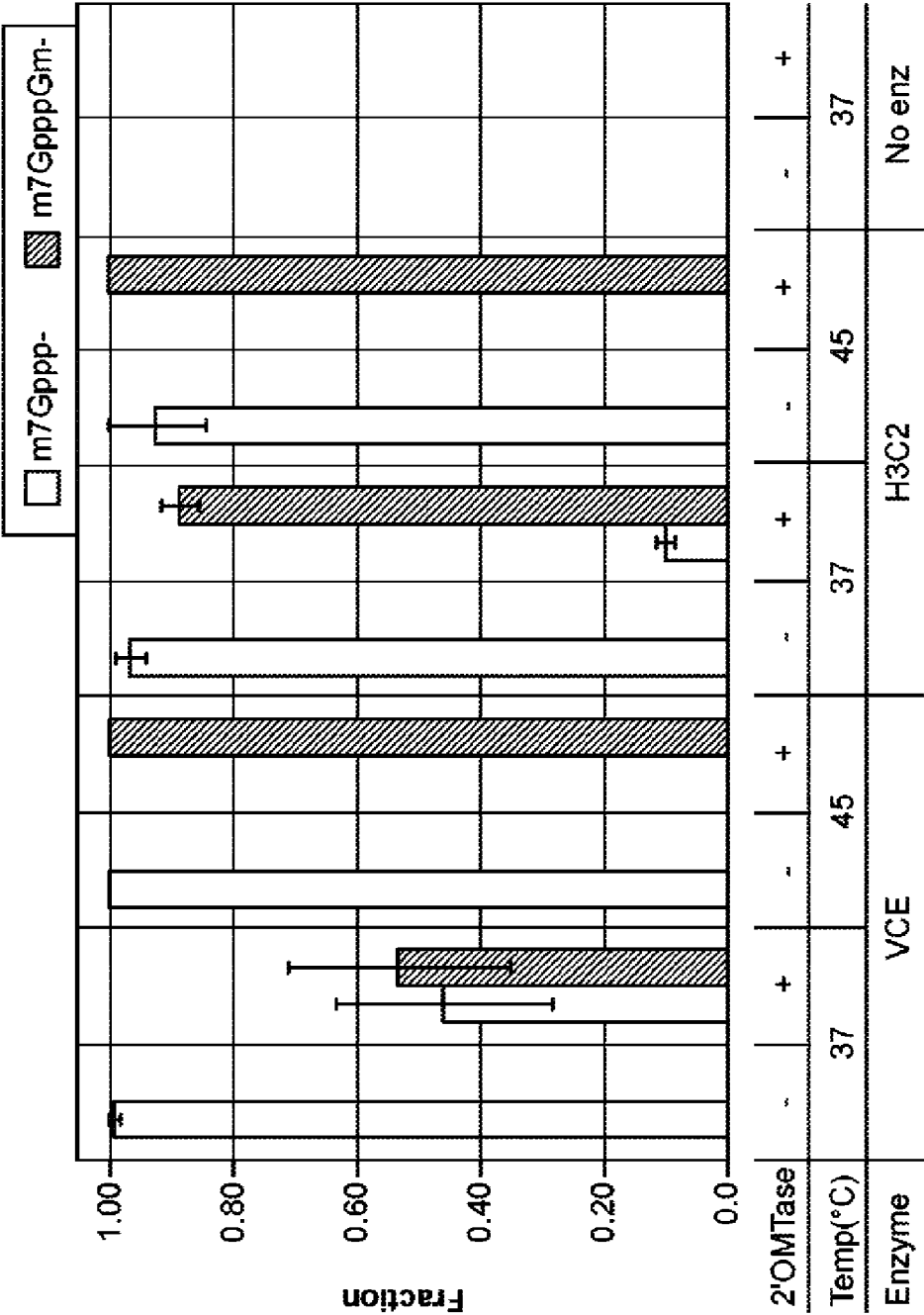
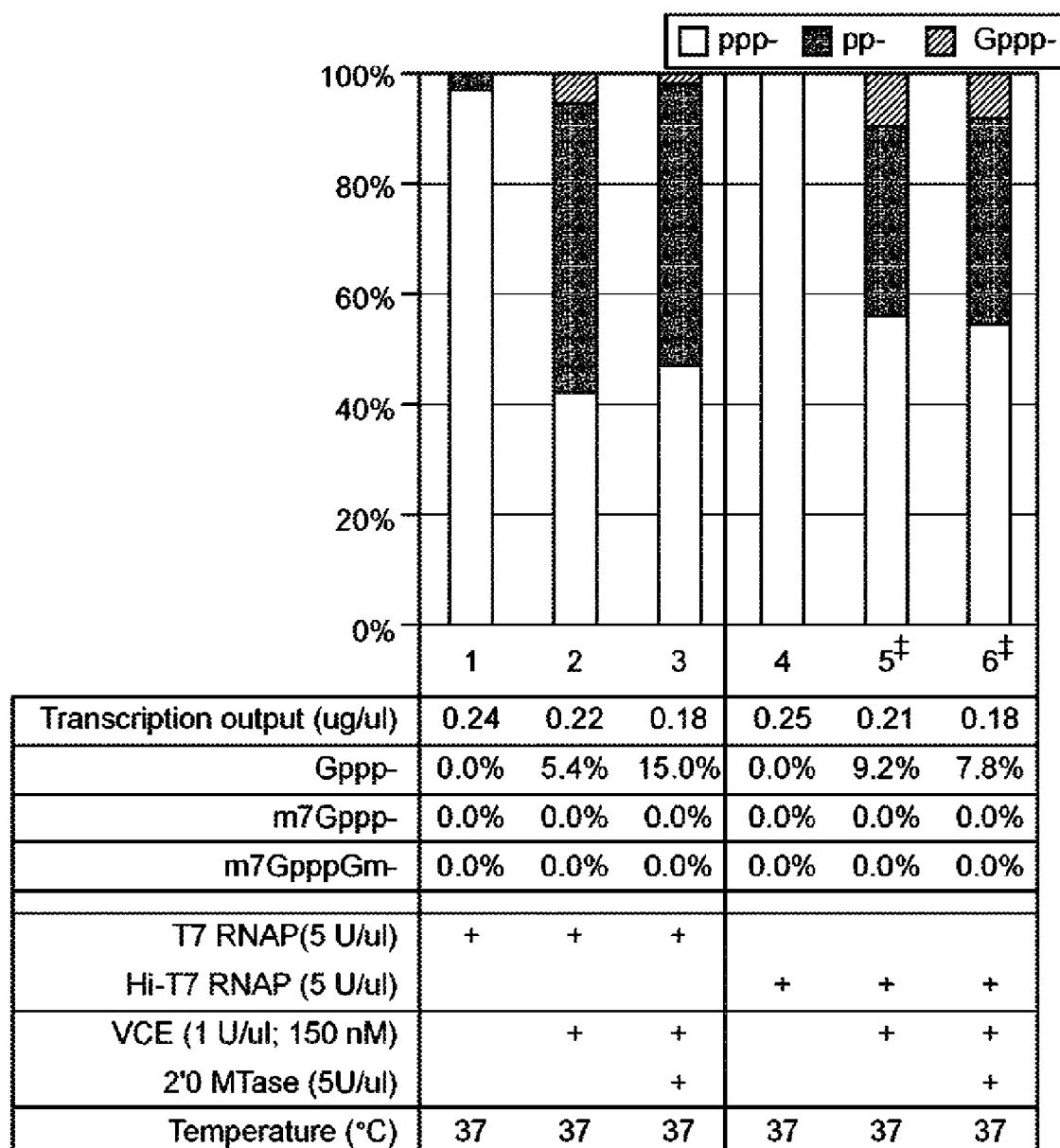


FIG. 11

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n = 3 unless stated otherwise

[‡]n = 2

FIG. 12

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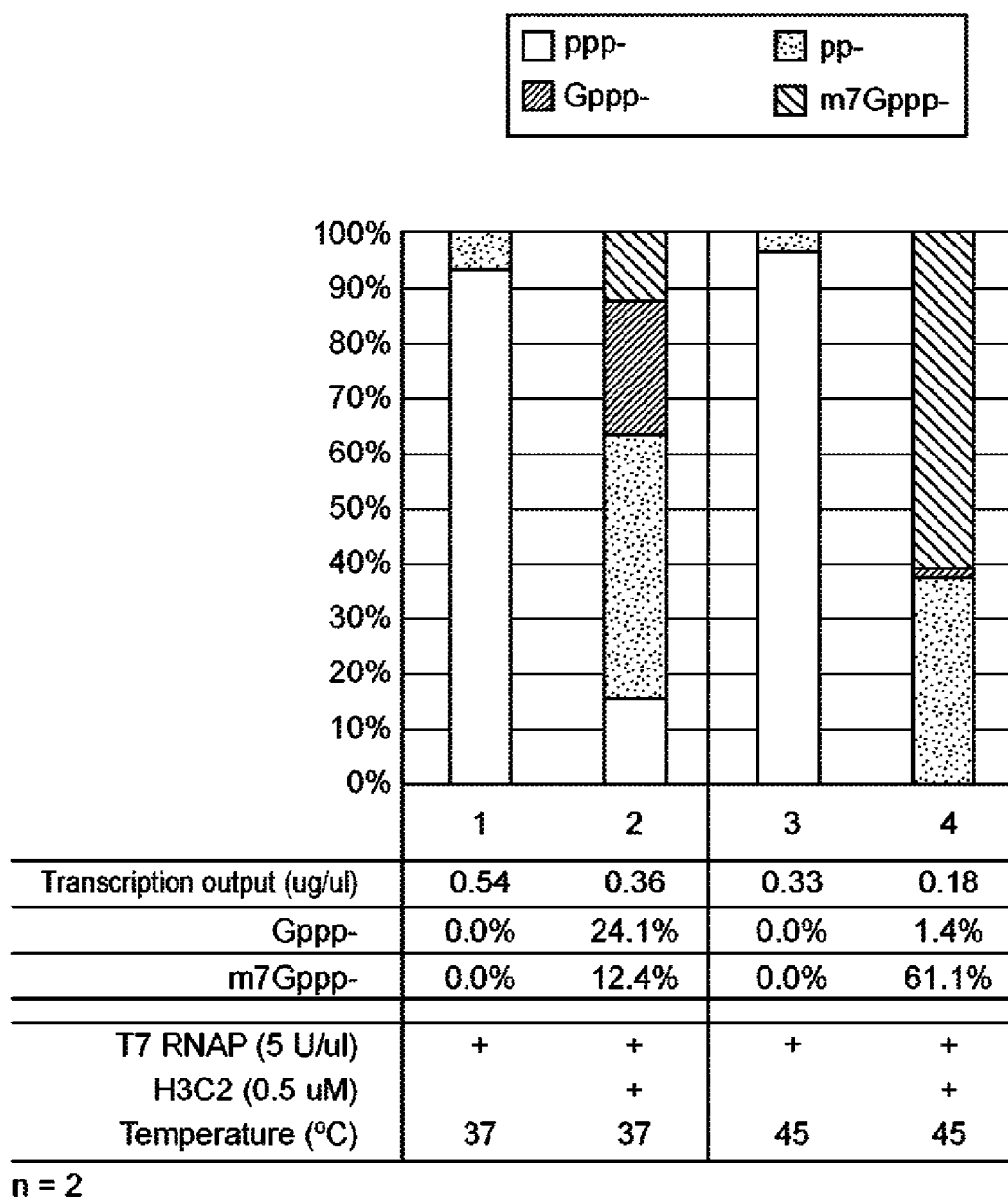
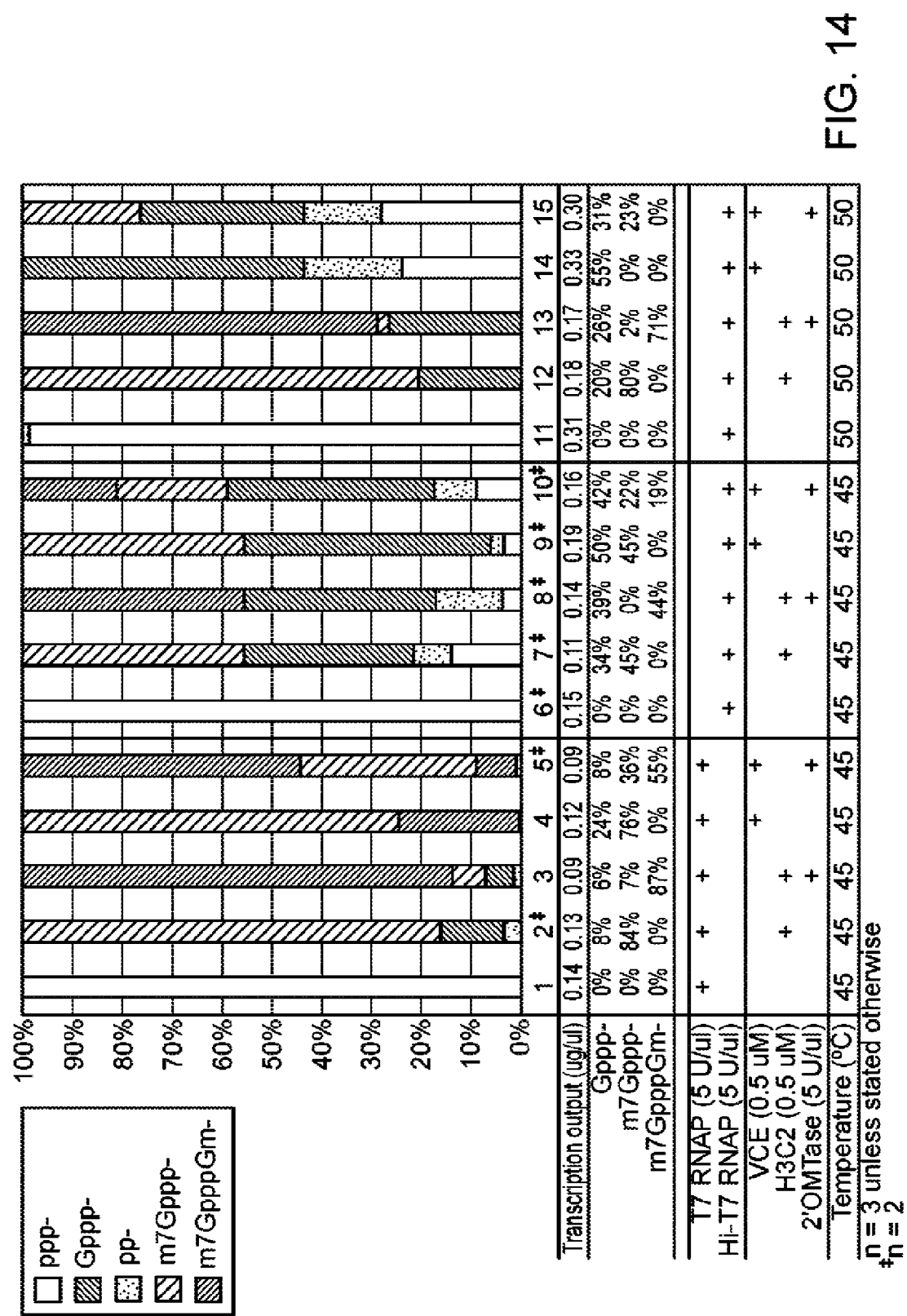
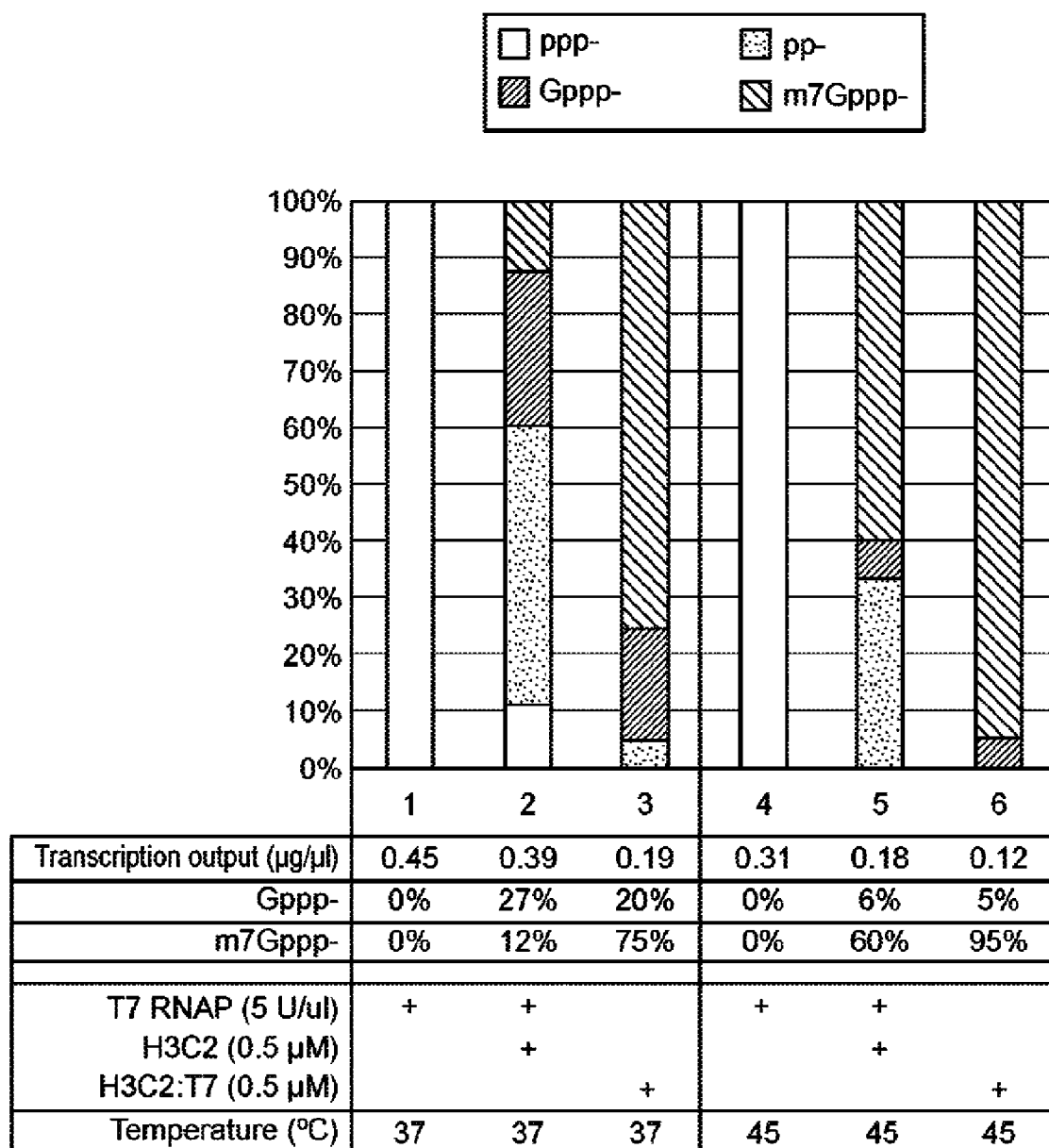


FIG. 13



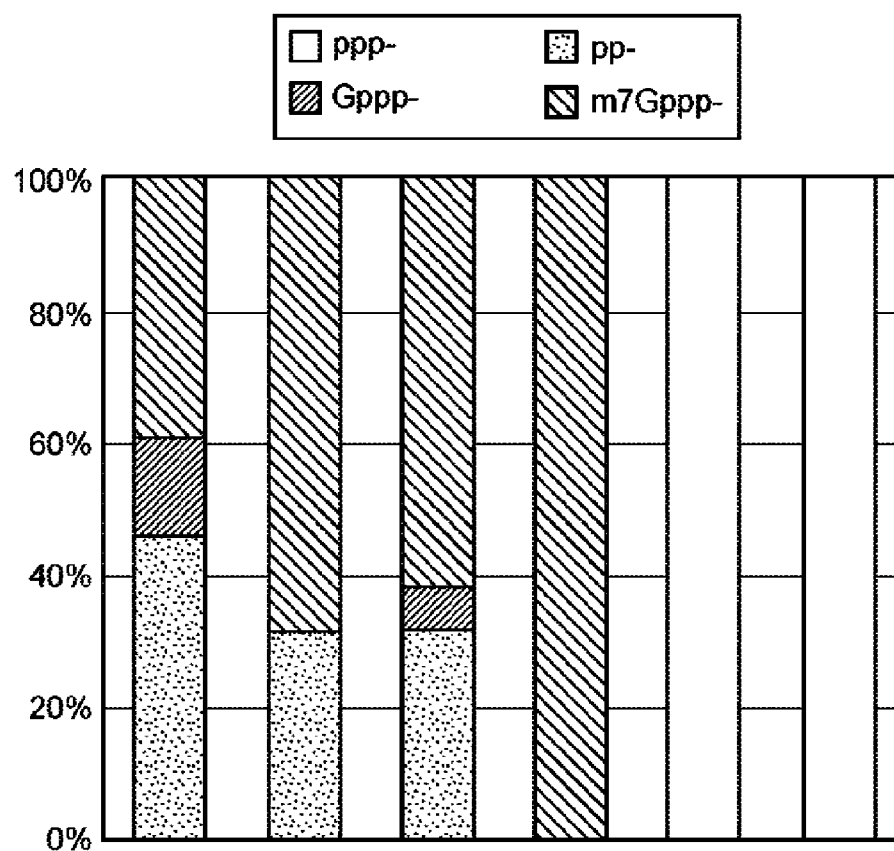
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n = 2

FIG. 15

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	1	2	3	4	5	6
Gppp-	15.0%	0.0%	6.2%	0.0%	0.0%	0.0%
m7Gppp-	38.0%	68.4%	61.4%	100.0%	0.0%	0.0%
rU	+	-	+	-	+	-
ψ	-	+	-	+	-	+
VCE	+	+	-	-	-	-
H3C2	-	-	+	+	-	-

FIG. 16

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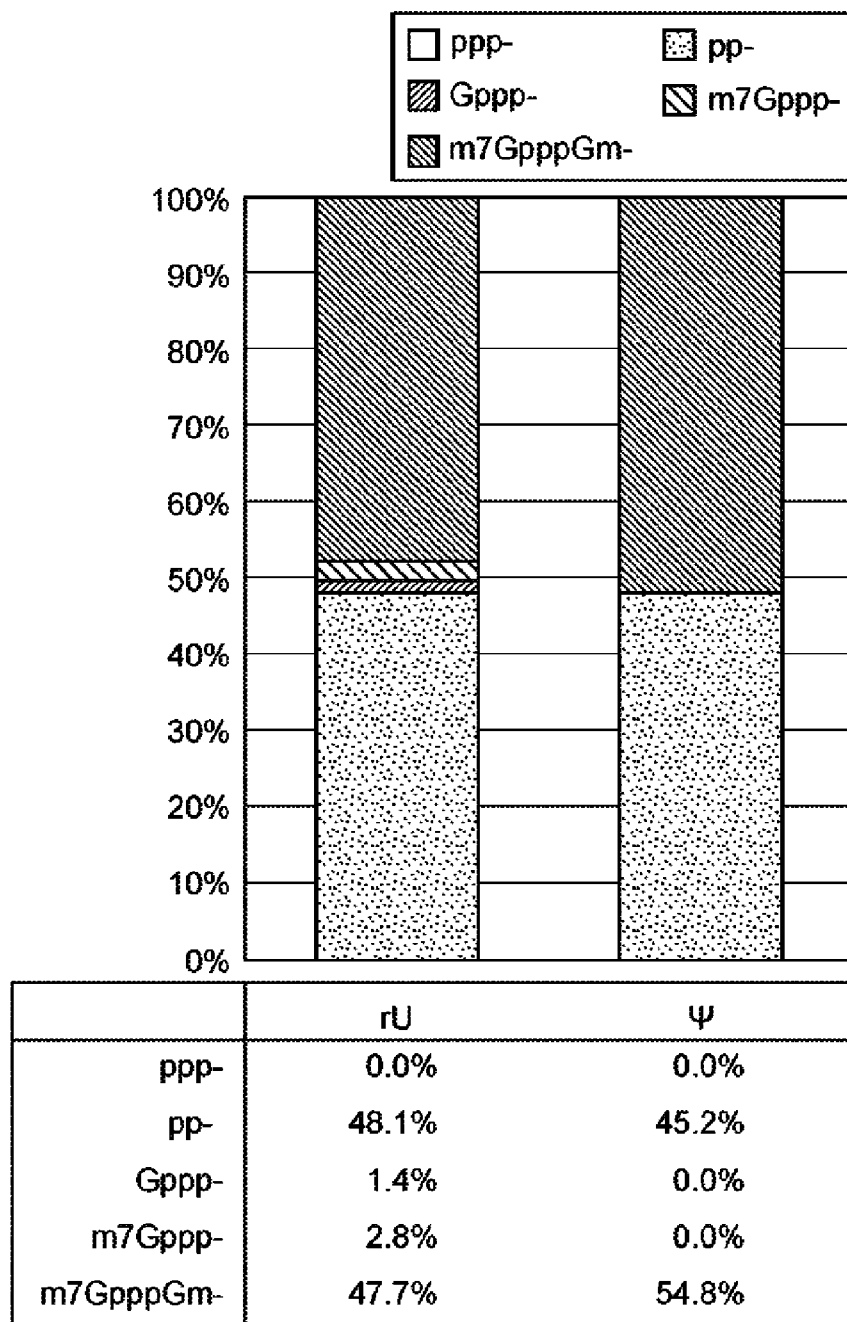


FIG. 17

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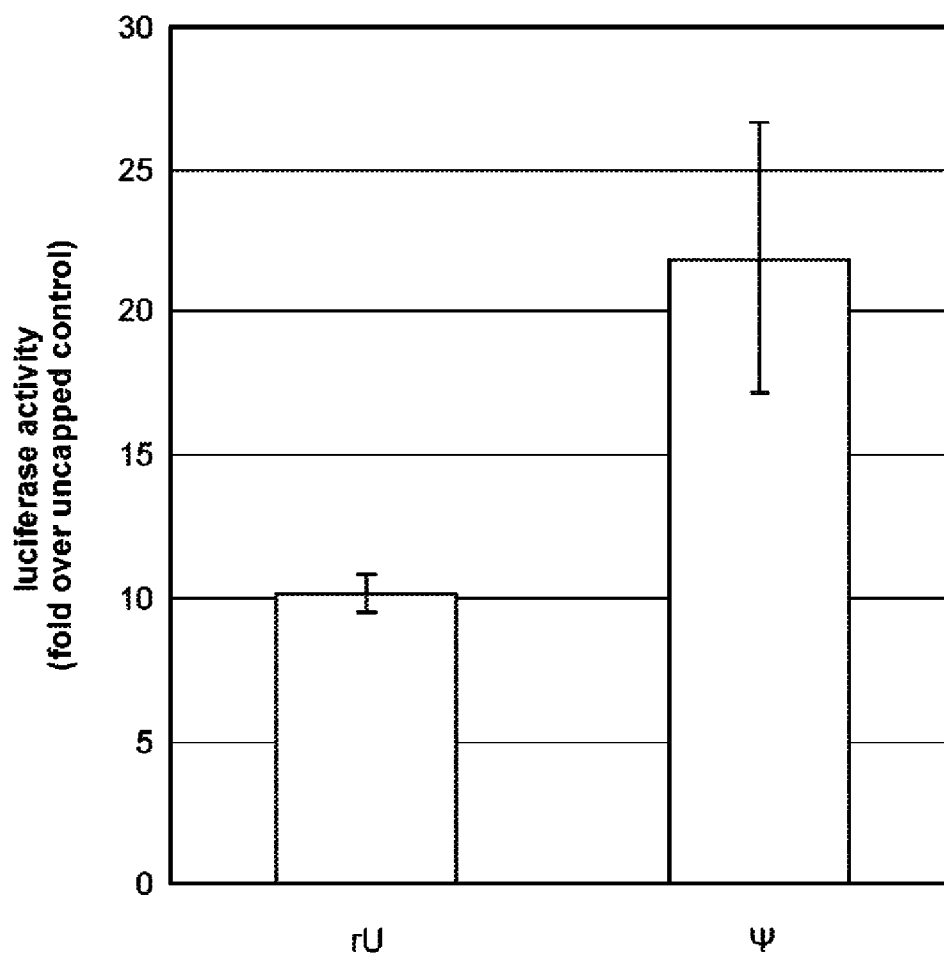


FIG. 18

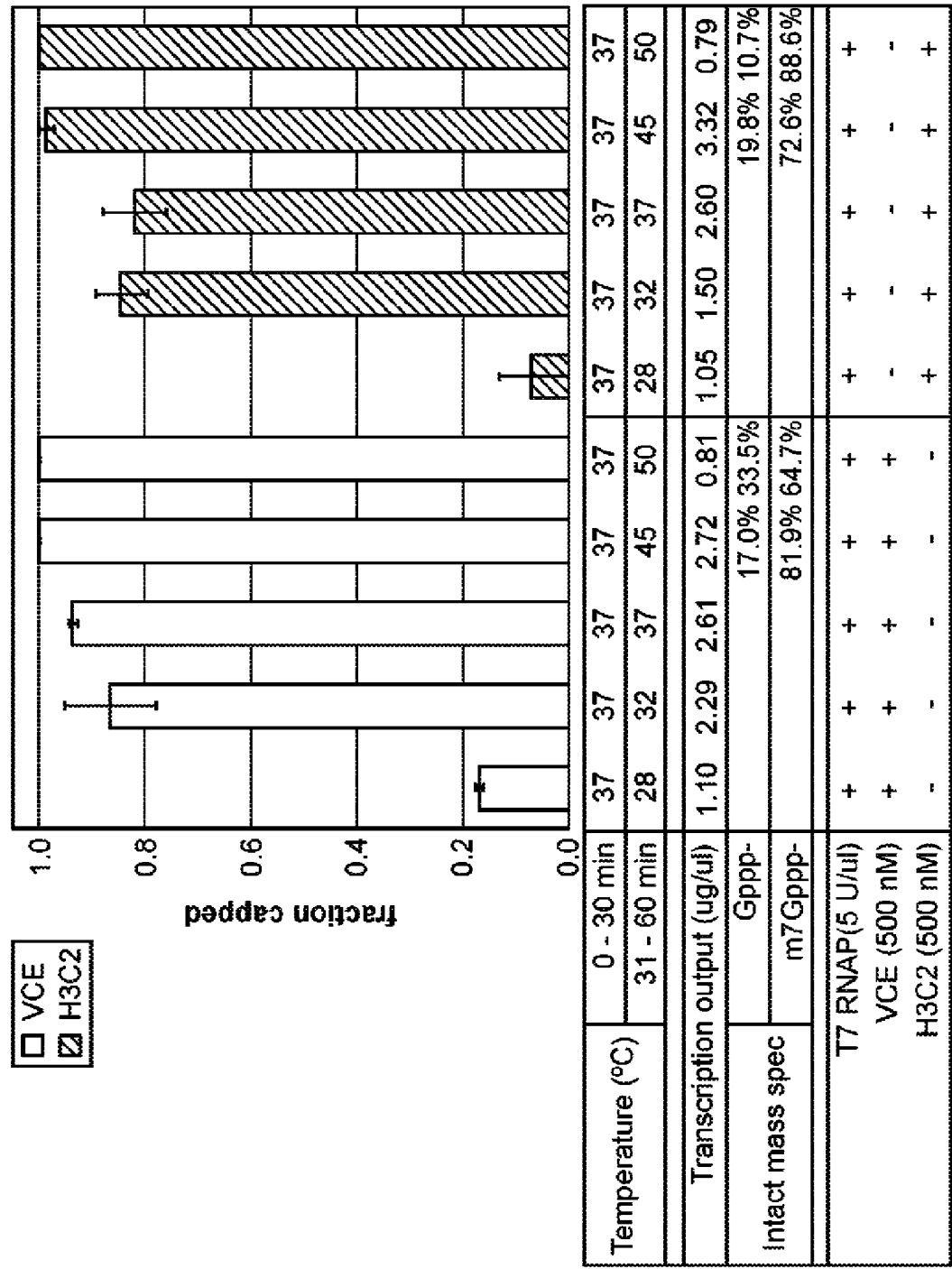


FIG. 19