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(71) Applicant: **THE SCRIPPS RESEARCH INSTITUTE** [US/US]; 10550 North Torrey Pines Road, La Jolla, California 92037 (US).

(72) Inventors: **ROMESBERG, Floyd**; 8109 Camino del Sol, La Jolla, California 92037 (US). **CHEN, Tingjian**; 3899 Nobel Drive, Apt. 1126, San Diego, California 92121 (US).

(74) Agent: **PHUKAN, Priti**; Umberg Zipser, LLP, 1920 Main Street, Suite 750, Irvine, California 92614 (US).

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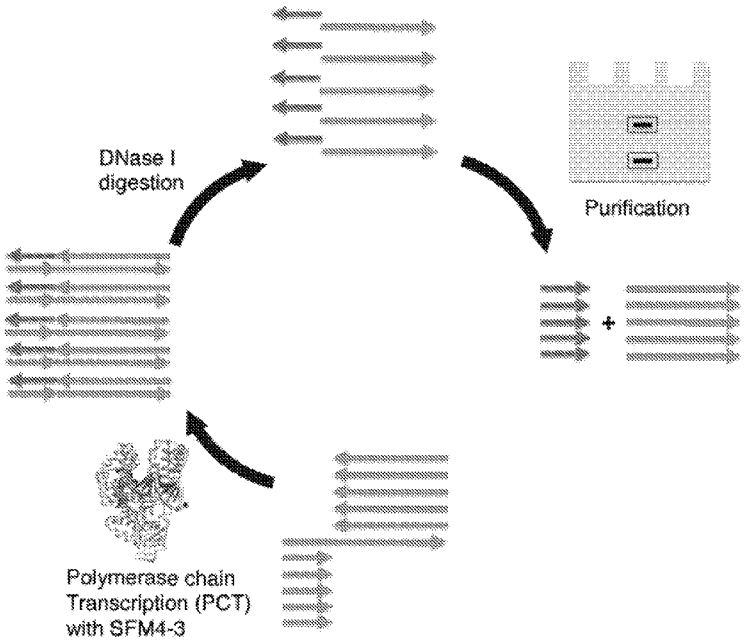
(54) Title: POLYMERASE CHAIN TRANSCRIPTION (PCT): EXPONENTIAL SYNTHESIS OF RNA AND MODIFIED RNA

Figure 11




(57) Abstract: Provided herein are methods of generating a RNA, comprising: providing a reaction mixture comprising a DNA template, a forward primer, a reverse primer, a SFM4-3 polymerase, and ribonucleotides; and generating the RNA by thermocycling the reaction mixture. Also provided herein are methods for generating a RNA or modified RNA at an elevated temperature, the method comprising: contacting a reaction mixture comprising a DNA template, a primer, a SFM4-3 polymerase, and ribonucleoside triphosphates; and generating the RNA by incubation at the elevated temperature or putting the reaction mixture through repeated cycles of heating and cooling. Further provided herein are kits for transcribing, synthesizing, and/or amplifying a RNA sequence comprising: a polymerase; and ribonucleoside triphosphates.

*[Continued on next page]*

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# POLYMERASE CHAIN TRANSCRIPTION (PCT): EXPONENTIAL SYNTHESIS OF RNA AND MODIFIED RNA

## CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of priority of U.S. Provisional Patent Application No. 62/531,603 filed on July 12, 2017, the contents of which are hereby incorporated by reference in its entirety.

## STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

The invention was made with government support under Grant No. N66001-14-2-4052 awarded by the Defense Advanced Research Projects Agency (DARPA). The U.S. government has certain rights in the invention.

## FIELD OF THE INVENTION

The present disclosure relates to methods and compositions for synthesis of RNA.

## BACKGROUND OF THE INVENTION

All publications herein are incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference. The following description includes information that may be useful in understanding the present invention. It is not an admission that any of the information provided herein is prior art or relevant to the presently claimed invention, or that any publication specifically or implicitly referenced is prior art.

RNA oligonucleotides have wide ranging applications in biomedical research, biotechnology, and the pharmaceutical industry. Although RNA oligonucleotides are commercially available through chemical synthesis, they are 10- to 15-fold more expensive than the corresponding DNA oligonucleotides. Thus, RNA is usually produced from the inexpensive ribotriphosphates via *in vitro* transcription using the RNA polymerase from T7 bacteriophage (T7 RNAP). However, while the transcription of longer RNA can result in the production of up to ~1000 transcripts per DNA template, shorter oligonucleotides are only inefficiently transcribed, resulting in the production of only ~10 to 250 transcripts per template. In all cases, efficient transcription requires a case-by-case optimization of the reaction conditions and the use of mM template concentrations. This problem is illustrated in Milligan, J. F. et al, Nucleic Acids Res. 1987, 15, 8783–8798, and the method disclosed in

the publication has been the standard method of generating RNA in the art. Moreover, transcription is inefficient with regards to NTPs, as up to half are wasted by incorporation into short abortive products. Finally, the use of T7 RNAP introduces sequence constraints, and because T7 RNAP is not thermostable, the transcription of templates with significant secondary structure can be problematic and PCR-like thermocycled transcription is not possible.

RNA with modified nucleotides, for example 2'-F-modifications, has attracted great interest due to its nuclease resistance, greater duplex stability, and unique and often better performance in in vivo applications (e.g. RNAi, CRISPR/Cas9-based genome editing). However, the chemical synthesis of 2'-F RNA is even more cost-prohibitive, with each base being ~200 times more expensive than its DNA analog. While transcription is possible with 2'-F modified nucleotides, it is less efficient and generally limited to CTP and UTP analogs.

Thus, there remains a need in the art for efficient and cost effective production of RNAs, modified RNAs, as well as other oligonucleotides.

## SUMMARY OF THE INVENTION

Various embodiments disclosed herein include a method of generating a RNA, comprising: providing a reaction mixture comprising a DNA template, a forward primer, a reverse primer, a SFM4-3 polymerase, and ribonucleotides; and generating the RNA by thermocycling the reaction mixture. In one embodiment, the method makes two different types of RNA sequences per DNA template. In one embodiment, the sequence of the DNA template is palindromic. In one embodiment, two RNA sequences are transcribed simultaneously by using a non-palindromic DNA template sequence. In one embodiment, concentration of the DNA template in the reaction mixture is between 0.1 nM and 20 nM. In one embodiment, the RNA is generated in an exponential manner. In one embodiment, the ratio of the RNA product to the DNA template used is about $10^2$, $10^3$, $10^4$, $10^5$, or $10^6$ times. In one embodiment, the method of generating a RNA comprises a method of transcribing RNA and/or a method of amplifying RNA. In one embodiment, the nucleotides are ribonucleotides, or modified ribonucleotides, or combinations thereof. In one embodiment, the thermocyling step comprises multiple cycles of steps a.-c.: (a) denaturating the reaction mixture by heating to about 90°C–95°C; (b) annealing the reaction mixture by cooling to about 35°C–50°C; and (c) transcribing the RNA sequence by heating to 45°C–55°C. In one embodiment, the RNA comprises modified RNA. In one embodiment, the RNA comprises 2'-modified RNA. In one embodiment, the RNA comprises 2'-F modified RNA. In one

embodiment, the RNA product comprises a DNA-RNA chimera. In one embodiment, the DNA of the DNA-RNA chimera is removed by incubation with a DNase. In one embodiment, the either or both of the primers are 3'-labeled with a ribonucleotide, which comprises phosphorylation modifications, attachment chemistry/linker modifications, biotinylation, fluorophores, dark quenchers, spacers, modified bases, phosphorothioate bonds modifications, and/or click chemistry modifications, to generate 5'-labeled RNA/modified RNA products after DNase digestion of the PCT products.

Various embodiments disclosed herein also include a method for generating a RNA or modified RNA at an elevated temperature, comprising: contacting a reaction mixture comprising a DNA template, a primer, SFM4-3 polymerase, and ribonucleotides; and generating the RNA or modified RNA at the elevated temperature or by putting the reaction mixture through repeated cycles of heating and cooling. In one embodiment, the RNA generated is a modified RNA. In one embodiment, the RNA generated is a 2'-modified RNA. In one embodiment, the RNA generated is a 2'-F modified RNA. In one embodiment, the nucleotides are dNTPs, rNTPs, modified dNTPs, modified rNTPs, or combinations thereof. In one embodiment, the elevated temperature is between about 45°C–55°C. In one embodiment, the primer is 3'-labeled with a ribonuleotide, which comprise phosphorylation modifications, attachment chemistry/linker modifications, biotinylation, fluorophores, dark quenchers, spacers, modified bases, phosphorothioate bonds modifications, and/or click chemistry modifications, to generate 5'-labled RNA/modified RNA products after DNase digestion of the transcription products.

Embodiments of the present disclosure further include a kit for transcribing, synthesizing, and/or amplifying a RNA sequence comprising: SFM4-3 polymerase; and ribonucleotides. In one embodiment, the kit further comprises dNTPs, modified dNTPs, modified rNTPs, or combinations thereof. In one embodiment, the modified dNTPs or rNTPs comprise phosphorylation modifications, attachment chemistry/linker modifications, biotinylation, fluorophores, dark quenchers, spacers, modified bases, phosphorothioate bonds modifications, and/or click chemistry modifications. In one embodiment, the ribonucleotides are isotopically labeled with $^{13}C$, $^{15}N$ and/or D isotopes. In one embodiment, the ribonucleotides comprises a 2' modified nucleotide, and/or an isotopically labeled nucleotide. In one embodiment, the kit further comprises a buffer. In one embodiment, the buffer comprises $MgCl_2$, Triton X-100, BSA, and Taq DNA polymerase buffer. In one embodiment, the kit further comprises instructions for using the kit.

Embodiments of the present disclosure also include a kit comprising a thermostable mutant DNA polymerase for generating RNA; and instructions for use, and wherein the use comprise use of ribonucleotides as substrate for the polymerase to generate RNA.

Other features and advantages of the invention will become apparent from the following detailed description, taken in conjunction with the accompanying drawings, which illustrate, by way of example, various embodiments of the invention.

## DESCRIPTION OF THE DRAWINGS

Exemplary embodiments are illustrated in referenced figures. It is intended that the embodiments and figures disclosed herein are to be considered illustrative rather than restrictive.

**Figure 1** depicts, in accordance with embodiments herein, SFM4-3 mediated RNA/2'-F-RNA synthesis and R/DNA PCR. **(A)** RNA transcription by SFM4-3. DNA template T1 (SEQ ID NO:1) (75 nt) was hybridized to a FAM-labeled DNA primer and transcribed by SFM4-3 or Sf. **(B)** 2'-F-C,U-RNA transcription by SFM4-3. DNA template T1 (SEQ ID NO:1) was hybridized to a FAM-labeled RNA primer and transcribed in the presence of ATP, 2'-F-CTP, GTP, and 2'-F-UTP by SFM4-3 or Sf. **(C)** Transcription of DNA template T7Ter-T (SEQ ID NO:6) containing a T7 terminator under non-thermocycling (NT) or thermocycling (T) conditions. **(D)** Fluorescence of DHFBI-1T bound to 1 μM Broccoli aptamer produced by T7 RNA polymerase (Bro), or T7-terminator-Broccoli aptamer produced by SFM4-3 from 1 μM primer (T7T-Bro). **(E)** qPCR curves of RNA/DNA PCR performed with 20 nM or 2 nM template T1 (SEQ ID NO:1). **(F)** R/DNA PCR products and digestion by NaOH. *h*, hybrid of biotinylated DNA template and R/DNA product; *p*, R/DNA PCR product; *d*, NaOH degradation product; SA, streptavidin.

**Figure 2** depicts, in accordance with embodiments herein, Polymerase Chain Transcription (PCT) of RNA and modified RNA. **(A)** Illustration of exponential production of chimeric DNA-RNA product by PCT. C1, C2, C3: cycle 1, 2, and 3. DNA, blue; RNA, red; primers indicated by half arrows. **(B)** qPCR curves of RNA PCT (20 cycles) with different concentrations of template T2 (SEQ ID NO:13) (0 nM–20 nM; Table 1) using DNA primers T2-F (SEQ ID NO:14) and T2-R (SEQ ID NO:15) (Table 1). **(C)** Different length RNA PCT products obtained using various templates of *n*+*m* composition: T5, 25+18 mer (SEQ ID NO:22); T2, 18+18 mer (SEQ ID NO:13); T3, 15+15 mer (SEQ ID NO:16); or T4, 12+12 mer (SEQ ID NO:19) using primers T2-F/T5-R (SEQ ID NO:14, 23), T2-F/R (SEQ ID NO:14-15), T3-F/R (SEQ ID NO:17-18), or T4-F/R, respectively (Table 1). M, DNA

ladder. **(D)** 2'-F-C,U-RNA PCT (20 cycles) with different concentrations of template T2 (SEQ ID NO:13) indicated (0 nM–20 nM; Table 1) using DNA primers T2-F (SEQ ID NO:14) and T2-R (SEQ ID NO:15) (Table 1). **(E)** Different length 2'-F-C,U-RNA PCT products obtained using the same template-primer combinations used in panel c. M: DNA ladder. **(F)** Release of RNA (left) or 2'-F-C,U-RNA (right) from PCT product by DNase degradation (25+18-mer template). **(G)** Thrombin binding of a 2'-F-C,U-RNA thrombin aptamer produced by PCT. Both unbound RNAs migrate as a single band at the bottom of the gel (8% PAGE). B: 2'-F-C,U-RNA bound with thrombin; U: 2'-F-C,U-RNA unbound. **(H)** Separation of 2'-F-C,U-RNA PCT products. The procedure is illustrated in Figure 13. Product, TurboDNase treated PCT product; Supernatant, fraction remaining after binding the product onto streptavidin beads, followed by wash steps and TurboDNase treatment; Eluant, fraction eluted from the beads; +Biotin, biotinylated 2'-FC, U-RNA strand; -Biotin, unbiotinylated 2'-F-C,U-RNA strand.

**Figure 3** depicts, in accordance with embodiments herein, transcription of RNA and 2'-F-modified RNA with SFM4-3. **(A)** RNA transcription fidelity test by primer extension. All transcription reactions were carried out with 500 nM primer/template, 1 μM SFM4-3 enzyme, and 0.5 mM each rNTPs in 1× standard Taq DNA polymerase buffer, and incubated at 50 °C for 12 h. **(B)** RNA transcription with a 2'-F-NTP replacing one of the four rNTPs (2'-F substituted analog is indicated along the top). All transcription reactions were carried out with 500 nM primer/template, 1 μM SFM4-3 enzyme, and 0.5 mM each rNTPs or 2'-F-NTPs in 1× standard Taq DNA polymerase buffer, and incubated at 50 °C for 12 h. **(C)** Secondary structure of T7 terminator-Broccoli fusion RNA transcript as predicted by mfold (Zuker, M. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. 31, 3406–3415 (2003).).

**Figure 4** depicts, in accordance with embodiments herein, fidelity of RNA transcription-reverse transcription PCR. **(A)** qPCR for integrated fidelity test of RNA transcription-reverse transcription-PCR. **1**, SFM4-3 enzyme was excluded in transcription; **2**, SuperScript III reverse was excluded in reverse transcription; **3**, both enzymes were excluded in transcription or reverse transcription, respectively; **4**, both enzymes were included in transcription or reverse transcription, respectively. **(B)** Gel assay of the PCR products for the integrated fidelity test of RNA transcription-reverse transcription-PCR (14 cycles of PCR). **(C)** qPCR for integrated fidelity test of 2'-F-C,U-RNA transcription-reverse transcription-PCR. Conditions **1**–**4** are the same as in panel A. **(D)** Gel assay of the PCR products for

integrated fidelity test of 2'-F-C,U-RNA transcription-reverse transcription-PCR (15 cycles of PCR).

**Figure 5** depicts, in accordance with embodiments herein, RNA PCR, R/DNA PCR and symmetric PCT. (**A**) RNA PCR with all four rNTPs. PCR was carried out with 20 nM template Biotin-T1 (SEQ ID NO:2), primers T1-F (SEQ ID NO:8) and T1-R (SEQ ID NO:3) (2 μM each), 1 mM each rNTPs, 0.1 % Triton X-100, 0.1% BSA, 200 nM SFM4-3 enzyme, and 2 mM extra $MgCl_2$ in 1 × standard Taq DNA polymerase buffer, and the following thermocycling conditions: 94 °C for 2 min; cycled 15×: 94 °C for 30 s, 49 °C for 1 min, 50 °C for 1 h; and final extension at 50 °C for 2 h. (**B**) RNase A digestion of RNA PCR product. Purified RNA PCR product was incubated with RNase A at 37 °C for 2 h, and then incubated with streptavidin at 37 °C for 1 h and assayed with PAGE gel. (**C**) R/DNA PCR with different combinations of dNTPs and rNTPs. R/DNA PCR was carried out with 20 nM template Biotin-T1 (SEQ ID NO:2), primers T1-F and T1-R (2 μM each), two dNTPs and two rNTPs (1 mM each), 0.1 % Triton X-100, 0.1% BSA, 200 nM SFM4-3 enzyme, and 1× standard Taq DNA polymerase buffer supplemented with 2 mM $MgCl_2$, and the following thermocycling conditions: 94 °C for 2 min; cycled 15×: 94 °C for 30 s, 49 °C for 1 min, 50 °C for 1 h; final extension at 50 °C for 2 h. (**D**) Fidelity test of R/DNA PCR with ATP, CTP, dGTP and dTTP. PCR conditions and thermocycling program used were the same as in panel **c**. A substantial amount of product was observed only when all four triphosphates were included. The absence of ATP, CTP, or both led to no product. *h*, hybrid of biotinylated DNA template and RNA product; *p*, RNA PCR product; *d*, RNA degradation product. (**E**) Symmetric PCT with a palindrome-like template T6 (SEQ ID NO:24) with primers T6-F (SEQ ID NO:25) and T6-R (SEQ ID NO:26) (Table 1).

**Figure 6** depicts, in accordance with embodiments herein, RNA/2'-F-C,U-RNA PCT/qPCT test of various concentrations of template T5 (25+18 mer) (SEQ ID NO:22). (**A**) RNA qPCT curves of 25+18 mer template T5 (SEQ ID NO:22) with various concentrations of template. (**B**) RNA PCT product of 25+18 mer template T5 (SEQ ID NO:22) with various concentrations of template. (**C**) 2'-F-C,U-RNA qPCT curves of 25+18 mer template T5 (SEQ ID NO:22) with various concentrations of template. (**D**) 2'-F-C,U-RNA PCT product of 25+18 mer template T5 (SEQ ID NO:22) with various concentrations of template. (**E**) 2'-F-C,U-RNA qPCT curves of 25+18 mer template T5 (SEQ ID NO:22) with various concentrations (narrow range) of template. (**F**) 2'-F-C,U-RNA PCT product of 25+18 mer template T5 (SEQ ID NO:22) with various concentrations (narrower range) of template. PCT/qPCT was carried out with a defined concentration of template, 400 nM SFM4-3 protein

(200 nM for 2'-F-C,U-RNA PCT), rNTPs or 2'-F-NTPs (1 mM each), 2 mM extra $MgCl_2$, 0.1 % Triton X-100, 0.01 % BSA, and 1× SYBR green I in 1× standard Taq DNA polymerase buffer, and with the following thermocycling program: initial denaturation of 94 °C for 30 s; 20 cycles of (94 °C, 15 s; 49 °C, 1 min; 50 °C, 1 h); final extension of 50 °C for 2 h.

**Figure 7** depicts, in accordance with embodiments herein, RNA/2'-F-C,U-RNA PCT/qPCT test of various concentrations of template T3 (18+18 mer) (SEQ ID NO:13). (**A**) RNA PCT product of 18+18 mer template T2 (SEQ ID NO:13) with various concentrations of template. (**B**) 2'-F-C,U-RNA PCT product of 18+18 mer template T2 (SEQ ID NO:13) with various concentrations of template. (**C**) 2'-F-A,G-RNA qPCT curves of 18+18 mer template T2 (SEQ ID NO:13) with various concentrations of template. (**D**) 2'-F-A,G-RNA PCT product of 18+18 mer template T2 (SEQ ID NO:13) with various concentrations of template. PCT/qPCT was carried out with defined concentration of template, 400 nM SFM4-3 protein (200 nM for 2'-F-C,U-RNA PCT), rNTPs or 2'-F-NTPs (1 mM each), 2 mM extra $MgCl_2$, 0.1 % Triton X-100, 0.01 % BSA, and 1 × SYBR green I in 1 × standard Taq DNA polymerase buffer, and with the following thermocycling program: initial denaturation of 94 °C for 30 s; 20 cycles of (94 °C, 15 s; 49 °C, 1 min; 50 °C, 1 h); final extension of 50 °C, 2 h.

**Figure 8** depicts, in accordance with embodiments herein, RNA/2'-F-C,U-RNA PCT/qPCT test of various concentrations of template T3 (15+15 mer) (SEQ ID NO:16). (**A**) RNA qPCT curves of 15+15 mer template T3 (SEQ ID NO:16) with various concentrations of template. (**B**) RNA PCT product of 15+15 mer template T3 (SEQ ID NO:16) with various concentrations of template. (**C**) 2'-F-C,U-RNA qPCT curves of 12+12 mer template T4 (SEQ ID NO:19) with various concentrations of template. (**D**) 2'-F-C,U-RNA PCT product of 15+15 mer template T3 (SEQ ID NO:16) with various concentrations of template. PCT/qPCT was carried out with defined concentration of template, 400 nM SFM4-3 protein (200 nM for 2'-F-C,U-RNA PCT), rNTPs or 2'-F-NTPs (1 mM each), 2 mM extra $MgCl_2$, 0.1 % Triton X-100, 0.01 % BSA, and 1× SYBR green I in 1× standard Taq DNA polymerase buffer, and with the following thermocycling program: initial denaturation of 94 °C for 30 s; 20 cycles of (94 °C, 15 s; 45 °C, 1 min; 50 °C, 1 h); final extension of 50 °C for 2 h.

**Figure 9** depicts, in accordance with embodiments herein, RNA/2'-F-C,U-RNA PCT/qPCT test of various concentrations of template T4 (12+12 mer) (SEQ ID NO:19). (**A**) RNA qPCT curves of 12+12 mer template T4 (SEQ ID NO:19) with various concentrations of template. (**B**) RNA PCT product of 12+12 mer template T4 (SEQ ID NO:19) with various concentrations of template. (**C**) 2'-F-C,U-RNA qPCT curves of 12+12 mer template T4 (SEQ

ID NO:19) with various concentrations of template. (**D**) 2'-F-C,U-RNA PCT product of 12+12 mer template T4 (SEQ ID NO:19) with various concentrations of template. PCT/qPCT was carried out with defined concentration of template, 400 nM SFM4-3 protein, rNTPs or 2'-F-NTPs (1 mM each), 2 mM extra $MgCl_2$, 0.1 % Triton X-100, 0.01 % BSA, and 1 × SYBR green I in 1 × standard Taq DNA polymerase buffer, and the following thermocycling program: initial denaturation of 94 °C for 30 s; 15 cycles of (94 °C, 15 s; 35 °C, 1 min; 50 °C, 1 h); final extension of 50 °C for 2 h.

**Figure 10** depicts, in accordance with embodiments herein, RNA/2'-F-C,U-RNA PCT/qPCT test of various concentrations of template T8 (50+18 mer) (SEQ ID NO:29). (**A**) RNA qPCT curves of 50+18 mer template T8 (SEQ ID NO:29) with various concentrations of template. (**B**) RNA PCT product of 50+18 mer template T8 (SEQ ID NO:29) with various concentrations of template. (**C**) 2'-F-C,U-RNA qPCT curves of 50+18 mer template T8 (SEQ ID NO:29) with various concentrations of template. (**D**) 2'-F-C,U-RNA PCT product of 50+18 mer template T8 (SEQ ID NO:29) with various concentrations of template. PCT/qPCT was carried out with a defined concentration of template, 400 nM SFM4-3 protein, rNTPs or 2'-F-NTPs (1 mM each), 2 mM extra $MgCl_2$, 0.1 % Triton X-100, 0.01 % BSA, and 1 × SYBR green I in 1 × standard Taq DNA polymerase buffer, and the following thermocycling program: initial denaturation of 94 °C for 30 s; 20 cycles of (94 °C, 15 s; 49 °C, 1 min; 50 °C, 1 h); final extension of 50 °C for 2 h.

**Figure 11** depicts, in accordance with embodiments herein, a procedure of using PCT to produce RNA/modified RNA oligonucleotides. Blue arrows indicate DNA template and primers; red and green arrows indicate different RNA products.

**Figure 12** depicts, in accordance with embodiments herein, production of 5'-labeled (modified) RNA. (**A**) Procedure of using PCT to produce 5'-labeled (modified) RNA. Blue arrows indicate DNA template and primers; red and green arrows indicate different RNA products. (**B**) Gel assay of 5'-labeled RNA generated via PCT. Lane 1: 3'-FAM-UTP-labeled DNA primer (19 mer) prepared by terminal transferase; lane 2: PCT product (44 bp) produced with 3'-FAM-UTP labeled primer; lane 3: 5'-FAM-labeled-RNA (26 mer) produced by degrading the DNA moiety in PCT product with TurboDNase.

**Figure 13** depicts, in accordance with embodiments herein, a procedure to rapidly separate/purify two different RNA/modified RNA molecules from PCT. Blue arrows: DNA; red arrows: RNA/modified RNA or ribonucleotide incorporated to the 3'-end of DNA primer; purple circles: biotin; SA: streptavidin beads.

## DETAILED DESCRIPTION

All references, publications, and patents cited herein are incorporated by reference in their entirety as though they are fully set forth. Unless defined otherwise, technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Hornyak, et al., Introduction to Nanoscience and Nanotechnology, CRC Press (2008); Singleton et al., Dictionary of Microbiology and Molecular Biology 3rd ed., J. Wiley & Sons (New York, NY 2001); March, Advanced Organic Chemistry Reactions, Mechanisms and Structure 7th ed., J. Wiley & Sons (New York, NY 2013); and Sambrook and Russel, Molecular Cloning: A Laboratory Manual 4th ed., Cold Spring Harbor Laboratory Press (Cold Spring Harbor, NY 2012), provide one skilled in the art with a general guide to many of the terms used in the present application. One skilled in the art will recognize many methods and materials similar or equivalent to those described herein, which could be used in the practice of the present invention. Indeed, the present invention is in no way limited to the methods and materials described.

As used herein, the term "nucleotide" refers to any ribonucleotide triphosphate and deoxyribonucleotide triphosphate with any natural or modified base in that structure that occurs in polymerized form as a component of a nucleic acid. The terms "rNTP" and "dNTP" refer to a ribonucleotide triphosphate and a deoxyribonucleotide triphosphate respectively with any natural or modified base. The terms "rNTPs" and "dNTPs" refer to a mixture of ribonucleotide triphosphates and deoxyribonucleotide triphosphates respectively consisting of at least two different ribonucleotide triphosphates or deoxyribonucleotide triphosphates. The term "NTP" is used to refer to a nucleoside triphosphate without reference to its specific sugar (e.g. a ribonucleoside triphosphate (rNTP), a deoxyribonucleoside triphosphate (dNTP), or a modified rNTP or dNTP).

The terms "polynucleotide," "oligonucleotide," and "nucleic acid" are used interchangeably herein, and refer generally to linear polymers of natural or modified nucleotides, including deoxyribonucleotides, ribonucleotides, alpha-anomeric forms thereof, and the like, usually linked by phosphodiester bonds or analogs thereof ranging in size from a few monomeric units, e.g. 2-4, to several hundreds of monomeric units. When a polynucleotide is represented by a sequence of letters, such as "ATGCCTG," it will be understood that the nucleotides are in 5'→3' order from left to right.

A variety of modifications may be incorporated into a nucleotide or polynucleotide, and use of such modified nucleotide or polynucleotide is contemplated by this disclosure.

Such modifications include, but are not limited to, phosphorylation modifications, attachment chemistry/linker modifications (such as acrydite, adenylation, azide, digoxigenin, cholesteryl-TEG, I-Linker, amino modifiers, alkynes, biotinylation, and/or thiol modifications), fluorophores, dark quenchers, spacers, modified bases, phosphorothioate bonds modifications, and/or click chemistry modifications. The nucleotides and/or polynucleotides disclosed herein may also be modified isotopically, such as labeling with $^{13}C$, $^{15}N$ and/or D isotopes. The compositions, methods, and reactions disclosed herein contemplate the use of modified, labeled, and/or unmodified nucleotides and/or polynucleotides.

As used herein, the term "RNA" refers to a nucleic acid molecule comprising at least one ribose sugar as opposed to a deoxyribose sugar as found in DNA. As used herein, RNA refers to all species of RNA including messenger RNA (mRNA), ribosomal RNA (rRNA), transfer RNA (tRNA) as well as small RNA species that have regulatory function. The term RNA as used herein may also refer to modified RNAs as well as DNA/RNA hybrids.

As used herein, the term "primer" refers to an oligonucleotide, synthetic or naturally occurring, which is capable of acting as a point of initiation of nucleic acid synthesis or replication along a template strand when placed under conditions in which the synthesis of a complementary strand is catalyzed by a polymerase. Within the context of transcription, primers are composed of nucleic acids and/or ribonucleic acids and prime on DNA templates. Within the context of reverse transcription, primers are composed of nucleic acids and prime on RNA templates. Within the context of PCR, primers are composed of nucleic acids and prime on DNA templates.

As used herein, the term "thermocycling" refers to the entire pattern of changing temperature most often used during an RT-PCR or PCR process. This process is common and well known in the art. See, for example, Sambrook supra; and U.S. Pat. No. 4,683,202 to Mullis et al. and U.S. Pat. No. 4,683,195 to Mullis et al. In general, thermocycling includes an initial denaturing step at high temperature, followed by a repetitive series of temperature cycles designed to allow template denaturation, primer annealing, and extension of the annealed primers by a polymerase.

As described herein, in accordance with the various embodiments herein, the inventors have developed a novel method, named polymerase chain transcription (PCT), that efficiently and cost-effectively produces large quantities of RNA sequences, including modified RNA sequences. In 2016, the instant inventors were interested in amplifying 2'-modified DNA, and for this purpose they generated several mutant forms of the Stoffel fragment of Taq DNA polymerase (See Chen, T. et al, Nat. Chem. 2016, 8, 556–562, which

is incorporated by reference herein in its entirety including the supplementary information). These mutant DNA polymerases were capable of more efficiently producing 2'-modified DNA compared to the wild form.

Surprisingly, as disclosed herein, the inventors found that one of these mutant DNA polymerases, which the inventors named SFM4-3, was also capable of transcribing RNA and modified RNA. As disclosed further herein, generation of RNA using the SFM4-3 polymerase solves many of the problems faced with conventional transcription using T7 RNAP. Unlike transcription with T7 RNAP, using SFM4-3 as the polymerase results in more efficient transcription, especially for shorter RNAs comprising ~10-250 transcripts; there are no sequence constraints, and allows for exponential production of RNAs, which is about $10^3$ to $10^5$ fold higher than conventional transcription with T7 RNAP.

In one embodiment, the inventors found that the thermostability of SFM4-3 allows for the transcription of templates that are difficult or impossible to otherwise transcribe, as well as the PCR amplification of R/DNA, and via PCT, the exponential production of large quantities of RNA or 2'-F modified RNA oligonucleotides from small quantities of DNA templates. Amplification levels with PCT (relative to the DNA template) were found to be $10^3$- to $10^5$- fold higher than those obtainable with conventional transcription (Table 4). In addition, PCT reduces the challenges associated with template secondary structure and sequence biases, and it also facilitates 5'-labeling. Thus, the inventors found that PCT is more efficient and general than conventional transcription, which would make accessible any RNA or modified RNA oligonucleotide on a scale previously only accessible via chemical synthesis, but at a fraction of the cost.

In one embodiment, disclosed herein is a polymerase chain transcription (PCT) reaction for transcribing, synthesizing, and/or amplifying a RNA sequence comprising: providing a DNA template sequence; contacting the DNA template sequence with a reaction mixture comprising a primer, a polymerase, and nucleotides; and transcribing, synthesizing, and/or amplifying the RNA sequence by thermocycling the reaction mixture. In one embodiment, the primer comprises a forward primer and a reverse primer. In one embodiment, the DNA template sequence is not a palindromic sequence. In one embodiment, two RNA sequences are transcribed simultaneously by using a non-palindromic DNA template sequence and two primers. In one embodiment, the polymerase is a thermophilic polymerase. In one embodiment, the polymerase is a variant of the Stoffel fragment of Taq DNA polymerase. In one embodiment, the polymerase is SFM4-3. In one embodiment, the thermocyling step comprises multiple cycles of steps a.-c.: (a) denaturing the reaction

mixture by heating to about 90 °C–95 °C; (b) annealing the reaction mixture by cooling to about 35 °C–50 °C; and (c) transcribing the RNA sequence by heating to 45 °C–55 °C. In one embodiment, the RNA sequence is transcribed at a temperature between 45 °C–46 °C, or 46 °C–47 °C, or 47 °C–48 °C, or 48 °C–49 °C, or 49 °C–50 °C, or 51 °C–52 °C, or 52 °C–53 °C, or 53 °C–54 °C, or 54 °C–55 °C. In one embodiment, the RNA sequence is a modified RNA sequence. In one embodiment, the RNA sequence is a 2'-modified RNA sequence. In one embodiment, the RNA sequence is a 2'-F modified RNA sequence.

An advantage of the PCT method is that it allows for the production of the RNA or the modified RNA at temperatures greater than 37 °C, where traditional transcription reactions are run. The elevated temperatures are typically about 40-65 °C, such as, for example about 40-65 °C, or more preferably about 42-62 °C, or more preferably about 44-59 °C, or more preferably about 46-56 °C, or more preferably about 48-53 °C, and most preferably about 50 °C. This facilitates the transcription of DNA with secondary structure or other properties that inhibit transcription at lower temperatures. An advantage of PCT thermocycling is that the transcription process may be converted to one that produces the RNA or modified RNA in an exponential process.

In one embodiment, disclosed herein is a method for transcribing a ribonucleic acid (RNA) sequence, the method comprising: contacting a reaction mixture comprising a DNA template, a primer, a SFM4-3 polymerase, and/or nucleotides (NTPs); and transcribing the RNA sequence by putting the reaction mixture through repeated cycles of heating and cooling. In one embodiment, the RNA sequence is a modified RNA sequence. In one embodiment, the RNA sequence is a 2'-modified RNA sequence. In one embodiment, the RNA sequence is a 2'-F modified RNA sequence. In one embodiment, the NTPs are dNTPs, rNTPs, and/or modified dNTPs or rNTPs.

In one embodiment, disclosed herein is a kit for transcribing, synthesizing, and/or amplifying a RNA sequence comprising: a polymerase; and nucleotides. In one embodiment, the nucleotides are dNTPs, rNTPs, and/or modified dNTPs or rNTPs. In one embodiment, the modified dNTPs or rNTPs comprise phosphorylation modifications, attachment chemistry/linker modifications, biotinylation, fluorophores, dark quenchers, spacers, modified bases, phosphorothioate bonds modifications, and/or click chemistry modifications. In one embodiment, the nucleotides are isotopically labeled with $^{13}C$, $^{15}N$ and/or D isotopes. In one embodiment, the nucleotide comprises a 2' modified nucleotide, and/or an isotopically labeled nucleotide. In one embodiment, the polymerase is a variant of the Stoffel fragment of Taq DNA polymerase. In one embodiment, the polymerase is SFM4-3. The inventors have

previously reported a Stoffel fragment of Taq polymerase (Sf) as an evolved and thermostable DNA polymerase. In one embodiment, a mutant of this polymerase, having the mutations V518A, N583S, I614E, E615G, D655N, E681K, E742Q, and M747R has been named SFM4-3. (See, Fa, M et al, Expanding the substrate repertoire of a DNA polymerase by directed evolution. J. Am. Chem. Soc. 2004; 126:1748–1754, Chen, T et al, "Evolution of Thermophilic DNA Polymerases for the Recognition and Amplification of C2'-Modified DNA," Nat Chem. 2016 June; 8(6): 556–562, and Chen, T et al Polymerase Chain Transcription: Exponential Synthesis of RNA and Modified RNA J. Am. Chem. Soc., 2017, 139 (29), pp 9949–9954, each of which are incorporated by reference herein in its entirety, including the figures and supplementary information). In one embodiment, the kit further comprises a buffer. In one embodiment, the buffer comprises $MgCl_2$, Triton X-100, BSA, and Taq DNA polymerase buffer.

Embodiments of the present disclosure also include a kit comprising a thermostable mutant DNA polymerase for generating RNA; and instructions for use, and wherein the use comprise use of ribonucleotides as substrate for the polymerase to generate RNA.

The kit is useful for practicing the inventive method of transcribing, synthesizing, and/or amplifying a polynucleotide sequence. The kit is an assemblage of materials or components, including at least one of the inventive compositions. Thus, in some embodiments the kit contains a composition including a polymerase, such as SFM4-3 and nucleotides, as described above. The exact nature of the components configured in the inventive kit depends on its intended purpose. For example, some embodiments are configured for the purpose of producing a modified RNA. In one embodiment, the kit is configured particularly for the purpose of amplifying RNA at a level about 1000-fold more than conventional transcription reaction using T7 RNAP. In another embodiment, the kit is configured particularly for the purpose of genetic research, prognosing/diagnosing and/or treating a disease in a mammal, forensic science, and environmental biology. In further embodiments, the kit is configured for research purposes, such as identification of new drug targets, drug screening studies and the like.

Instructions for use may be included in the kit. "Instructions for use" typically include a tangible expression describing the technique to be employed in using the components of the kit to effect a desired outcome, such as to amplify an oligonucleotide. Optionally, the kit also contains other useful components, such as, diluents, buffers, pharmaceutically acceptable carriers, syringes, catheters, applicators, pipetting or

measuring tools, bandaging materials or other useful paraphernalia as will be readily recognized by those of skill in the art.

The materials or components assembled in the kit can be provided to the practitioner stored in any convenient and suitable ways that preserve their operability and utility. For example the components can be in dissolved, dehydrated, or lyophilized form; they can be provided at room, refrigerated or frozen temperatures. The components are typically contained in suitable packaging material(s). As employed herein, the phrase "packaging material" refers to one or more physical structures used to house the contents of the kit, such as inventive compositions and the like. The packaging material is constructed by well-known methods, preferably to provide a sterile, contaminant-free environment. The packaging materials employed in the kit are those customarily utilized in the medical and bio-pharmaceutical field. As used herein, the term "package" refers to a suitable solid matrix or material such as glass, plastic, paper, foil, and the like, capable of holding the individual kit components. The packaging material generally has an external label which indicates the contents and/or purpose of the kit and/or its components.

Embodiments of the present disclosure are further described in the following examples. The examples are merely illustrative and do not in any way limit the scope of the invention as claimed.

## EXAMPLES

*Example 1*

*RNA transcription by SFM4-3*

The inventors first explored the ability of SFM4-3 to accept ribotriphosphates and transcribe RNA at 50 °C, the temperature at which it was evolved. DNA template T1 (SEQ ID NO: 1) (75 nt) was hybridized to a FAM-labeled primer and transcribed by SFM4-3, which yielded full-length product which was virtually devoid of shorter oligonucleotides (Figure 1A). As expected, under identical conditions wild type Sf produced no product. Next, the inventors examined the ability of SFM4-3 to synthesize RNA with 2'-F modified nucleotides by transcribing template T1 (SEQ ID NO:1) under the same conditions described above but using 2'-F modified purine or pyrimidine triphosphates. In each case, full-length product was again observed, but not in controls with the parental Sf polymerase (Figure 1B and 3B).

*Example 2*

*Fidelity of natural and modified RNA synthesis by SFM4-3*

To characterize the fidelity with which SFM4-3 synthesizes natural or 2'-F modified RNA, a 90-nt template, T-L (SEQ ID NO:9, Table 1), was transcribed into RNA or 2'-F-C,U-RNA, and the product was incubated with TurboDNase (to remove DNA template and primer), before being reverse transcribed back into DNA with Superscript III reverse transcriptase (see Example 9, Figure 4). The DNA produced was analyzed by qPCR with Q5 DNA polymerase or by PCR and PAGE, which revealed efficient product formation, but not in control reactions lacking SFM4-3 or Superscript III (Figure 4). Sequencing of the amplified DNA (see Example 9, Figure 4) revealed that the major mutations in both cases were A to G transitions (Table 3) and a mutation frequency for the combined transcription-reverse transcription-PCR of $2 \times 10^{-3}$ for RNA (which is only ~30-fold reduced relative to T7 RNAP-mediated transcription) and $1.2 \times 10^{-2}$ for 2'-F-C,U-RNA. This data suggests that the fidelity of SFM4-3 is likely sufficient for many practical applications. For example, as described below, the RNA aptamer Broccoli and the 2'-F-RNA thrombin aptamer produced by SFM4-3 are both functional.

**Table 1**: Oligonucleotides

| | | |
|---|---|---|
| T1 | SEQ ID NO:1 | 5'-CTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCC |
| Biotin-T1 | SEQ ID NO:2 | 5'-Biotin-CTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCC |
| T1-R | SEQ ID NO:3 | 5'-GGCTTTACACTTTATGCTTCCG |
| FAM-T1-R | SEQ ID NO:4 | 5'-FAM-TGGCTTTACACTTTATGCTTCCG |
| FAM-T1-R-RNA | SEQ ID NO:5 | 5'-FAM-rUrGrGrCrUrUrUrArCrArCrUrUrUrArUrGrCrUrUrCrCrG |
| T7Ter-T[a] | SEQ ID NO:6 | 5'-AGTTCCTCCTTTCAGCAAAAAACCCCTCAAGACCCGTTTAGAGGCCCCAAGGGGTTATGCTAGTTATTCGGAAGCATAAAGTGTAAAGCC |
| T7T-Broccoli - | SEQ ID NO:7 | 5'-CATCTGAGCCCACACTCTACTCGACAGATACGAAT |

| | | |
|---|---|---|
| T[a,b] | | ATCTGGACCCGACCGTCTCAGATGATCCAAAAAAC CCCTCAAGACCCGTTTAGAGGCCCCAAGGGGTTAT GCTAGCGGAAGCATAAAGTGTAAAGCC |
| T1-F | SEQ ID NO:8 | 5'-CTGTTTCCTGTGTGAAATTGTTAT |
| T-L | SEQ ID NO:9 | 5'-CTGTTTCCTGTGTGAAATTGTTATGAACTCAACGAC ATTCCGCTCACAATTCCACACAACATACGAGCCGG AAGCATAAAGTGTAAAGCC |
| T1-CL-F | SEQ ID NO:10 | 5'-ATGATCATGAATTCCTGTTTCCTGTGTGAAATTGTT AT |
| T1-CL-R | SEQ ID NO:11 | 5'-ATAGAATTAAGCTTGCTCGTATGTTGTGTGGA |
| pUC19-seq | SEQ ID NO:12 | 5'-GGGGGATGTGCTGCAAGGCG |
| T2 (18+18 mer) | SEQ ID NO:13 | 5'-AAATTGTTATCCGCTCACTCCACACAACATACGAG C |
| T2-F | SEQ ID NO:14 | 5'-AAATTGTTATCCGCTCAC |
| T2-R | SEQ ID NO:15 | 5'-GCTCGTATGTTGTGTGGA |
| T3 (15+15 mer) | SEQ ID NO:16 | 5'-TTGTTATCCGCTCACTCCACACAACATACG |
| T3-F | SEQ ID NO:17 | 5'-TTGTTATCCGCTCAC |
| T3-R | SEQ ID NO:18 | 5'-CGTATGTTGTGTGGA |
| T4 (12+12 mer) | SEQ ID NO:19 | 5'-TTATCCGCTCACTCCACACAACAT |
| T4-F | SEQ ID NO:20 | 5'-TTATCCGCTCAC |
| T4-R | SEQ ID NO:21 | 5'-ATGTTGTGTGGA |
| T5 (25+18 mer) | SEQ ID NO:22 | 5'-GGGTAAGTACTTCAGCTTTGTTCCCGTGAGCGGAT AACAATTT |
| T5-R | SEQ ID NO:23 | 5'-GGGTAAGTACTTCAGCTTTGTTCCC |
| T6 | SEQ ID NO:24 | 5'-AAATTGTTATCCGCTCACCCACAGTGAGCGGATAA CAATTT |
| T6-F | SEQ ID NO:25 | 5'-AAATTGTTATCCGCTCACCCACA |
| T6-R | SEQ ID NO:26 | 5'-AAATTGTTATCCGCTCACTGTGG |
| T7 | SEQ ID NO:27 | 5'-GGGTAAGTACTTCAGCTTTGTTCCCAGTGAGCGGA TAACAATTT |
| T7-R | SEQ ID NO:28 | 5'-GGGTAAGTACTTCAGCTTTGTTCCCA |
| T8 (50+18 mer) | SEQ ID NO:29 | 5'-CTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGAT TTTTGTGATGCTCGGTGAGCGGATAACAATTT |
| T8-R | SEQ ID NO:30 | 5'- |

| | | |
|---|---|---|
| | | CTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCG |
| T7-25-F | SEQ ID NO:31 | 5'-TAATACGACTCACTATAGGGAACAAAGCUGAAGUACUUACCC |
| T7-25-R | SEQ ID NO:32 | 5'-GGGTAAGTACTTCAGCTTTGTTCCCTATAGTGAGTCGTATTA |
| T7-18-F | SEQ ID NO:33 | 5'-TAATACGACTCACTATAGGGACACAACATACGAGC |
| T7-18-R | SEQ ID NO:34 | 5'-GCTCGTATGTTGTGTCCCTATAGTGAGTCGTATTA |
| T7-15-F | SEQ ID NO:35 | 5'-TAATACGACTCACTATAGGGACACAACATACG |
| T7-15-R | SEQ ID NO:36 | 5'-CGTATGTTGTGTCCCTATAGTGAGTCGTATTA |
| T7-12-F | SEQ ID NO:37 | 5'-TAATACGACTCACTATAGGGACACAACAT |
| T7-12-R | SEQ ID NO:38 | 5'-ATGTTGTGTCCCTATAGTGAGTCGTATTA |
| T7P-Bro-F | SEQ ID NO:39 | 5'-TAATACGACTCACTATAGGGCATCTGAGACGGTCGGGTCC |
| T7P-Bro-R | SEQ ID NO:40 | 5'-CATCTGAGCCCACACTCTACTCG |

[a.] T7 terminator sequence:
CAAAAAACCCCTCAAGACCCGTTTAGAGGCCCCAAGGGGTTATGCTAG
[b.] Broccoli aptamer sequence:
GAGCCCACACTCTACTCGACAGATACGAATATCTGGACCCGACCGTCTC

*Example 3*

*Thermostability of SFM4-3*

The utility of the thermostability of SFM4-3 was examined by transcribing the T7Ter-T template (SEQ ID NO:6), which contains a T7 RNAP terminator formed by a 7-nt palindrome that folds into a stem-loop structure followed by four uridines (Table 1) and which represents an efficient block to conventional transcription. Under the same conditions described above, a 12-h incubation yielded full-length product, but also a significant level of shorter transcripts (Figure 1C); however, three cycles of 50 °C for 3 h followed by 72 °C for 1 h, yielded full-length product and no detectable shorter fragments (Figure 1C). To further demonstrate the utility of thermos-stability and that the RNA produced is functional, the inventors examined the transcription of a DNA template that fuses the T7 terminator to the 3' end of DNA encoding the Broccoli aptamer (5' end in the transcript) (SEQ ID NO:39-40). (Table 1 and Figure 3C). The transcription product was incubated with TurboDNase and folded, and the fluorescence observed upon addition of the aptamer's fluorophore, DFHBI-

1T, was comparable to that observed with the same broccoli aptamer produced by conventional T7 RNAP transcription (Figure 1D). This demonstrates that SFM4-3 is able to transcribe through a T7 terminator and produce the functional aptamer.

*Example 4*

*Ability of SFM4-3 to amplify RNA*

The ability of SFM4-3 to PCR-amplify RNA was explored. With a variety of DNA templates and the natural ribotriphosphates, thermocycling resulted in the production of significant quantities of RNA, which was confirmed by degradation with RNase A (Figure 5A, B). However, analysis by qPCR revealed that the amplification was not exponential, presumably due to the added challenge of efficiently recognizing the ribonucleotides as triphosphates as well as in the primer and template. To reduce these challenges, PCR amplification of template T1 (SEQ ID NO: 1) with different combinations of deoxy- and ribotriphosphates were done. Exponential production of R/DNA (mixed DNA and RNA) was observed, with the presence of the ribonucleotides in the product confirmed by NaOH degradation; in contrast, no product was observed in control reactions with Sf (Figure 1E, F). The most efficient amplification was observed with A, C, dG, and dT, or A, dC, G, and dT (Figure 5C).

*Example 5*

*Polymerase Chain Transcription (PCT)*

To explore an alternate route to the exponential production of RNA, the inventors examined the use of a nicked DNA duplex, with the nicked strand added in excess. In this format, each nicked strand acts as a primer for RNA synthesis using the intact strand as a template. However, each nicked strand also binds to the complementary RNA sequence of the RNA-DNA chimera produced and primes additional transcription reactions. Thus, unlike normal transcription, this process, referred to as polymerase chain transcription (PCT), has the potential to produce product exponentially. The inventors' first efforts focused on palindromic templates so that each primer extends to form the same RNA. However, no amplification product was observed, which they reasoned might result from poor primer binding due to the formation of template dimers (which have much greater lengths of complementarity than do the primer binding regions) and/or from template hairpin formation. While templates with additional sequence introduced between the palindromes were amplified, the amplification was not efficient (Figure 5E).

Thus, the inventors explored "asymmetric PCT", wherein the intact template is not palindromic and extension of the two primers thus produces two different RNAs (Figure 2A). Various concentrations of template strands of different length, designated as n+m where n and m refer to the length of the two RNAs transcribed, were subjected to PCT. The DNA-RNA chimeric product was exponentially produced with as little as 300 nM enzyme and 0.2 nM template, except with the 12+12 template (SEQ ID NO:19), which required 2 nM template (Figure 2B and Figures 6–9). Slight decreases in fluorescence were observed in the qPCT curves at long times, which is likely due to SYBR green I dye instability. Product was confirmed by PAGE (Figure 2C and Figures 6–9) and the desired RNA oligonucleotide was easily obtained by incubation with TurboDNase (Figures 2F and 11). In each case, faint additional bands are observed, which likely result from the addition of extra A nucleotides to the terminus, an activity present with SFM4-3's parental enzyme, Sf. Amplification levels (relative to DNA template) of $10^3$ to $10^4$-fold were observed (Table 4). For comparison, the inventors also transcribed the four corresponding templates with T7 RNAP. Consistent with previously reported studies, amplification levels of 10- to 20- fold were observed (Table 2), confirming the significantly greater efficiency of PCT. RNA PCT was also possible with longer templates, for example a 50+18 template (SEQ ID NO:29), although it was less efficient (Figure 10A and 10C).

**Table 2**: T7 RNAP transcription of small RNAs (for comparison with PCT reactions)

| Template (Table S1) | Length of transcript (nts) | Transcript copy generated per template |
|---|---|---|
| T7-25-F/R | 25 | 20 |
| T7-18-F/R | 28 | 14 |
| T7-15-F/R | 15 | 13 |
| T7-12-F/R | 12 | 21 |

**Table 3:** Mutation bias in the fidelity test of transcription-RT-PCR of RNA and 2'-F-RNA (in template sequence)

| RNA (988 nts sequenced; 2 mutations observed) | |
|---|---|
| Mutation type | Occurrence times |
| A→G | 2 |
| 2'-F-RNA (1014 nts sequenced; 12 mutations observed) | |
| Mutation type | Occurrence times |

| | |
|---|---|
| A→G | 7 |
| C→A | 2 |
| A→C | 1 |
| T→C | 1 |
| Deletion of C | 1 |

**Table 4**: Amplification of RNA or 2' –F-C, U-RNA

| Template size (n+m, bp) | Template concentration (nM) | Fold amplification[a] |
|---|---|---|
| RNA PCT | | |
| 25+18 | 0.2 | $1.5 \times 10^4$ |
| 18+18 | 0.2 | $1.2 \times 10^4$ |
| 15+15 | 0.2 | $7.4 \times 10^3$ |
| 12+12 | 2 | $7.8 \times 10^2$ |
| | | |
| 2' –F-C, U-RNA PCT | | |
| 25+18 | 0.02 | $1.1 \times 10^5$ |
| 18+18 | 0.02 | $8.6 \times 10^4$ |
| 15+15 | 0.02 | $5.0 \times 10^4$ |
| 12+12 | 0.2 | $8.3 \times 10^3$ |

[a] = Ratio of product to template

*Example 6*

*PCT of modified RNA*

To explore the PCT of modified RNA, analogous experiments were performed in which CTP and UTP were replaced with 2'-F-CTP and 2'-F-UTP. The exponential generation of the PCT product was apparent with concentrations of SFM4-3 and template as low as 200 nM and 0.02 nM, respectively, except with the 12+12 template, which still only required 0.2 nM template (Figure 4 and Figures 6–9). Product was confirmed by PAGE (Figure 2E and Figures 6–9) and amplification levels (relative to DNA template) of $10^4$ to $10^5$-fold were observed (Table 1). The PCT of 2'-F-A,G RNA, which is not accessible via conventional transcription, was also efficient (Figures 7C, D). As with RNA PCT, the PCT of 2'-F-C,U-RNA with longer templates was possible, but less efficient (Figure 10B and 10D).

The desired 2'-F modified RNA oligonucleotides were straightforwardly obtained by digestion with TurboDNase (Figure 10). To demonstrate the function of a modified RNA PCT product, the inventors PCT-amplified with the same triphosphates template T5 (SEQ ID NO:22) (Table 1), which encodes a 2'-F-C,U-modified thrombin aptamer. Product was purified as described above, and was shown to retain function via a thrombin gel shift assay (Figure 2G).

*Example 7*

*PCT to produce 5'-labeled oligonucleotides*

To explore the use of PCT to produce 5'-labeled oligonucleotides, which are difficult to produce by conventional transcription, a similar nicked duplex was employed, but in this case one primer strand terminated with a FAM-labeled ribonucleotide (5-FAM-X-U) (Figure 12A). After PCT with ATP, GTP, 2'-F-CTP, and 2'-F-UTP, the product was treated with TurboDNase to produce the 5'-FAM-labled 2'-F-C,U-RNA, the efficient production of which was confirmed by PAGE and scanning with Typhoon scanner (FAM channel) (Figure 12B). To demonstrate the use of a 5'-label to purify a PCT product, a similar nicked duplex was employed, but in this case one primer strands terminated with a biotin-11-U (Figure 13). After PCT, the biotinylated PCT product was incubated with streptavidin beads, washed, and then subjected to treatment with TurboDNase to degrade the DNA and release the first (unlabeled) 2'-F-C,U-RNA oligonucleotide. After additional washing, the second (5'-biotin labeled) 2'-F-C,U-RNA oligonucleotide was eluted from the beads by incubation in 98% formamide supplemented with 10 mM EDTA (pH=8.0) at 90 °C for 10 min. Recovery of each 2'-F-C,U-labeled oligonucleotide was confirmed by PAGE (Figure 2H).

*Example 8*

*Advantages of PCT*

There is ever-increasing need for the cheap production of oligonucleotides, and while this has largely been achieved for DNA, the production of RNA or modified RNA oligonucleotides remains less efficient and significantly more expensive. As disclosed herein, the inventors have shown that SFM4-3 is capable of the efficient transcription of RNA and 2'-F-RNA. While Holliger and coworkers have reported the selection of a mutant Tgo DNA polymerase that is capable of transcribing RNA, (See Cozens, C. et al, Proc. Natl. Acad. Sci. USA 2012, 109, 8067-8072), SFM4-3 represents the first example of thermophilic family-A DNA polymerase mutant that can efficiently transcribe RNA. The thermostability of SFM4-3

allows for the transcription of templates that are difficult or impossible to otherwise transcribe, as well as the PCR amplification of R/DNA, and via PCT, the exponential production of large quantities of RNA or 2'-F RNA oligonucleotides from small quantities of DNA templates. Amplification levels with PCT (relative to the DNA template) are $10^3$- to $10^5$- fold higher than those obtainable with conventional transcription. In addition, PCT reduces the challenges associated with template secondary structure and sequence biases, and it also facilitates 5'-labeling. Thus, PCT is more efficient and general than conventional transcription and should make accessible any RNA or modified RNA oligonucleotide on a scale previously only accessible via chemical synthesis, but at a fraction of the cost. While the fidelity of PCT is somewhat reduced relative to conventional transcription, it should be sufficient for many practical applications, especially to quickly and cheaply explore the activity of multiple oligonucleotides, with the best then prepared via chemical synthesis if higher fidelity is required. While, in some embodiment, the DNA of the DNA-RNA chimeras initially produced by PCT were removed, its presence may prove useful for different applications, including purification or as the 'sticky bridge' of oligonucleotide assemblies. While the current implementation of PCT requires the production of two different oligonucleotides per DNA template, this is not a disadvantage in many applications, for example when two or more oligonucleotides are desired, such as when evaluating siRNAs or candidate aptamers. Nonetheless, efforts toward the optimization of PCT with palindromic substrates are currently in progress. Finally, the broad substrate tolerance of SFM4-3 suggests that modifications other than 2'-F are likely to be accommodated.

*Example 9*

*Materials and methods*

I. Materials. Ribonucleoside triphosphates (rNTPs), deoxyribonucleoside triphosphates (dNTPs), Q5 Hot Start DNA polymerase, T7 RNA polymerase, and terminal transferase (TdT) were obtained from New England Biolabs (Ipswich, MA). 2'-Fluoro-2'-deoxyribonucleoside triphosphates (2'-F-NTPs) were obtained from TriLink Biotechnologies (San Diego, CA). Biotin-11-UTP was obtained from Biotium (Fremont, CA). 5-FAM-X-UTP was obtained from GeneCopoeia (Rockville, MD). (Z)-4-(3,5-difluoro-4-hydroxybenzylidene)-2-methyl-1-(2,2,2-trifluoroethyl)-1H-imidazol-5(4H)-one (DHFBI-1T) was obtained from Lucerna (Brooklyn, NY). SYBR gold, SYBR green I, SuperScript III reverse transcriptase, TurboDNase, and Dynabeads MyOne Streptavidin C1 magnetic beads were obtained from Thermo Fisher Scientific (Waltham, MA). DNA and RNA

oligonucleotides were obtained from Integrated DNA Technologies (San Diego, CA). Human a-thrombin protein was obtained from Haematologic Technologies (Essex Junction, VT). Zymo ssDNA/RNA purification kits were obtained from Zymo Research (Irvine, CA). Qiaquick Nucleotide Removal Kit was obtained from Qiagen (Hilden, Germany). Centrifugal filtration was accomplished with Amicon devices obtained from EMD Millipore (Darmstadt, Germany). Thermocycling was accomplished with an MJ Research PTC-200 DNA Engine, or in the case of reactions containing SYBR green I, with a CFX Connect Real-Time PCR Detection System (Bio-Rad; Hercules, CA). Fluorescence was measured in a 96-well plate format with an EnVision 2103 Multilabel Reader (PerkinElmer).

II. Expression and purification of SFM4-3 polymerase. Expression and purification of Stoffel fragment mutant SFM4-3 were carried out as described previously.12 Briefly, plasmid pET23b-SFM4-3 was transformed into E. coli BL21 (DE3)/pLysS via electroporation. A single colony was inoculated into LB or 2×YT medium supplemented with 100 μg/mL ampicillin and 34 μg/mL chloramphenicol, and grown with shaking at 37 °C overnight. The overnight culture was then diluted 1:100 into fresh LB or 2×YT and grown with shaking at 30 °C until $OD_{600}$ reached 0.4–0.6, at which time 0.4 mM isopropyl-D-thiogalactoside (IPTG) was added to induce expression of polymerase. The culture was then transferred to room temperature and grown with shaking overnight. Cells from the resulting culture were collected by centrifugation, and lysed by sonication. The cell lysate was incubated at 70 °C for 30 min to denature cellular proteins. The supernatant was then collected, and subjected to nickel affinity chromatography and ion exchange (DEAE) chromatography. The resulting purified protein was then dialyzed into 50 mM Tris-HCl (pH 8.5), 0.5 mM EDTA, concentrated with an Amicon Ultra Centrifugal Filter (MWCO 30 kDa), and stored at - 20 °C as a 50% glycerol solution.

III. Primer extension and transcription of RNA or modified RNA. Generally, 1 μM FAM-labeled DNA primer FAM-T1-R was annealed to 2 μM DNA template T1 (SEQ ID NO:1) in 2× standard Taq DNA polymerase buffer using the following thermocycling program: 95 °C, 5 min; 0.1 °C/s to 25 °C; incubate on ice, 5 min. The annealed product (500 nM) was then mixed with 1 μM SFM4-3, and 0.5 mM each rNTPs or rNTPs with one or two replaced with their 2'-F-modified analogs in 1× standard Taq DNA polymerase buffer. The reaction was incubated at 50 °C for defined times (up to 12 h), or subjected to the following thermocycling transcription program: 3 x (50 °C, 3 h; 72 °C, 1 h); 50 °C, 3 h. The reaction was then quenched by the addition of 2 volumes of quenching buffer (95% formamide, 18 mM EDTA, 0.025% SDS, xylene cyanol and bromophenol blue), and heated to 98 °C for 10

min. The product was then analyzed on an 18% denaturing PAGE gel (supplemented with 8 M urea), and scanned with a Typhoon 9410 scanner (GE Amersham Molecular Dynamics).

IV. Functional test of the RNA product generated by SFM4-3 and transcription of a difficult sequence (T7 terminator). Template T7T-Broccoli-T (4 mM) (SEQ ID NO:7) (Table 1) was annealed to 2 mM DNA primer T1-R (SEQ ID NO:3) (Table 1) in 2× Standard Taq DNA polymerase buffer (NEB) using follow program: 95 °C, 5 min; 0.1 °C/s to 25 °C; incubate on ice, 5 min. The annealed product (25 mL) was mixed with rNTPs (0.5 mM each) and SFM4-3 (1 mM) enzyme in a total volume of 50 mL. The following thermocycling program was performed: 3 x (50 °C, 3 h; 72 °C, 1 h); 50 °C, 3 h. To each reaction, 5 mL 10′ TurboDNase buffer and 2.5 mL TurboDNase were added, and the resulting mixtures were incubated at 37 °C for 1 h to digest DNA primers and templates. The RNA products were then purified with the Zymo ssDNA/RNA purification kit, and each product was eluted into 40 mL DNase/RNase free water. For preparation of the broccoli aptamer with T7 RNA polymerase, a transcription template was prepared by PCR with T7T-Broccoli-T (SEQ ID NO:7) as the template and T7P-Bro-F (SEQ ID NO:39) and T7P-Bro-R (SEQ ID NO:40) as primers (Table 1). The PCR product was purified by spin column (Zymo DNA purification kit), and subjected to transcription with T7 RNA polymerase according to the manufacturer's instructions. The DNA template was then removed by incubating the product with TurboDNase, and the RNA transcript was purified by spin column (Zymo ssDNA/RNA purification kit). RNA concentration was determined with a Qubit fluorometer using the reagents and assay parameters for RNA. A 10-mL aliquot of 5′ binding buffer (100 mM HEPES, pH 7.5, supplemented with 750 mM NaCl, 30 mM KCl, and 10 mM $MgCl_2$) was then added to each RNA product (40 mL of 1.25 mM stock solution), and the RNA was folded by heating at 75 °C for 5 min, and rapidly cooling down on ice. To each folded-RNA solution, DHFBI-1T was added to a final concentration of 200 mM. As a control, 200 mM DHFBI-1T was added to 50 mL 1 x binding buffer (20 mM HEPES, pH 7.5, supplemented with 150 mM NaCl, 6 mM KCl, and 2 mM $MgCl_2$). The experimental and control solutions were incubated at room temperature for 10 min to allow RNA and DHFBI-1T to bind. The solutions were then diluted with 150 mL 1x binding buffer, and transferred into 96-well black polystyrene microwell plates. The fluorescence was then measured using a plate reader ($l_{ex}$=485; $l_{em}$=535). All experiments were performed in triplicate.

V. Integrated fidelity of transcription-RT-PCR. The longer template T-L (SEQ ID NO:9) (90 nt, Table 1) was annealed to DNA primer P1, and subjected to a transcription reaction mediated with SFM4-3 as described above with rNTPs, or ATP, GTP, 2'-F-CTP and

2'-F-UTP. The transcription products were then incubated with TurboDNase at 37 °C for 2 h to remove the DNA tem-plate and primer, and after adding 20 mM EDTA into the reaction, TurboDNase was inactivated by heating the reaction to 75 °C for 30 min. The RNA or 2'-F-RNA products were then purified with a Zymo ssDNA/RNA column, and annealed to reverse primer T1-F in a solution containing dNTPs (1 mM each) by heating to 65 °C for 5 min, and rapidly cooling on ice. The annealed products were then mixed with 1x SuperScript III buffer, 5 mM $MgCl_2$, 10 mM DTT, and SuperScript III reverse transcriptase (1 mL for 20 mL reaction), and dNTP (0.5 mM each). The reverse transcription reaction was incubated at 50 °C for 1–2 h. For control reactions, SFM4-3, or Super-Script III, or both were not included in transcription or/and reverse transcription respectively. The transcription-reverse transcription products were then analyzed by qPCR with Q5 Hot Start DNA polymerase, and the PCR products were analyzed by PAGE gel to confirm that the product is reverse transcribed from the transcription product mediated by SFM4-3. The transcription-reverse transcription products were then amplified using Q5 Hot Start DNA polymerase with cloning primers T1-CL-F (SEQ ID NO:10) and T1-CL-R (SEQ ID NO:11) (Table 1). The products were then purified, digested with EcoRI-HF and HindIII-HF, purified again, inserted into digested vector pUC19, transformed into E. coli XL1-Blue cells, and plated onto LB plates supplemented with X-gal, IPTG, and ampicillin. Positive clones were picked for sequencing.

VI. PCR and qPCR of RNA and R/DNA. Template T1 (SEQ ID NO:1) or Biotin-T1 (SEQ ID NO:2) (2–20 nM) was mixed with primers T1-F and T1-R (2 mM each), two dNTPs and two rNTPs, or four rNTPs (1 mM each), 0.1% Triton X-100, 0.01% bovine serum albumin (BSA), and 200 nM SFM4-3 enzyme in 1× standard Taq DNA polymerase buffer supplemented with 2 mM $MgCl_2$. For qPCR, reactions were also supplemented with 1× SYBR green. The following thermocycling program was performed for R/DNA (or RNA) PCR or qPCR: 94 °C, 2 min; 10–20 cycles of (94 °C, 30 s; 49 °C, 1 min; 50 °C, 1 h); 50 °C, 2 h. The products were analyzed with a native PAGE gel. Product fraction containing the biotin-labeled template was visualized by gel assay of PCR products (5-10 μL) that had been incubated with excess amount of streptavidin (2 μL of 1 mg/mL).

VII. Degradation test of R/DNA (mixed DNA and RNA) or RNA PCR product. The R/DNA or RNA PCR products were purified with the Zymo ssDNA/RNA purification kit, supplemented with 100 mM NaOH, and incubated at 65 °C for 30 min. The reaction was then neutralized with 100 mM HCl, and incubated with streptavidin at 37 °C for 1 h (to demonstrate the fraction containing biotin-labeled DNA template). The products were then analyzed by PAGE.

VIII. Polymerase chain transcription (PCT) and quantitative PCT (qPCT) of RNA or modified RNA. Forward and reverse primers (2 μM each, as defined in different PCT reaction) were mixed with 0.02–20 nM template, 50–400 nM SFM4-3 protein, rNTPs or 2'-F-NTPs (1 mM each), 2 mM extra $MgCl_2$, 0.1% Triton X-100 and 0.01% BSA in 1× standard Taq DNA polymerase buffer. For qPCT, 1× SYBR green I was also added. The PCT reaction was then subjected to the following program: 94 °C, 0.5–2 min; 10–20 cycles of (94 °C, 15-30 s; 35-49 °C, 1 min; 50 °C, 1 h). qPCT progress was monitored by tracking the fluorescence with a qPCR instrument (Bio-Rad), and all PCT products were assayed with PAGE. The concentration of the PCT product was measured with a Qubit fluorometer (Thermo Fisher Scientific) using the reagents and assay option for dsDNA.

IX. Transcription of templates with T7 RNAP. Transcription templates were prepared by annealing the oligonucleotides T7-25-F/T7-25-R (SEQ ID NO:31-32), T7-18-F/T7-18-R (SEQ ID NO:33-34), T7-15-F/T7-15-R (SEQ ID NO:35-36), or T7-12-F/T7-12-R (SEQ ID NO:37-38) (Table 1), respectively, using the following thermocycling program: 95 °C, 5 min; 0.1 °C/s to 25 °C; incubate on ice, 5 min. Transcription with T7 RNA polymerase was then carried out according to the manufacturer's instructions with optimal template concentration (2 mM) for the longest recommended transcription time (16 h). DNA templates in the resulting transcription product were removed via incubation with TurboDNase. The concentrations of RNA products were then determined with a Qubit fluorometer (Thermo Fisher Scientific) using the reagents and assay parameters for RNA, and the ratio of RNA product and DNA template was calculated.

X. Generation and purification of RNA from PCT product. For analysis of the PCT product and demonstration of RNA generation with a small amount of PCT product, 1× TurboDNase buffer and TurboDNase were added directly into the PCT reaction, and the mixture was incubated at 37 °C, the product was then directly analyzed with PAGE. For larger scale PCT, one biotinylated primer was used in the PCT reaction. The PCT product was incubated with magnetic streptavidin beads at 37 °C for 2 h. The beads were then washed 3–6 times with BWBS buffer (10 mM Tris•HCl pH 7.4, 1 M NaCl, 0.1% Tween20, 1 mM EDTA), and the RNA product was cleaved off the beads by incubating with TurboDNase at 37 °C for 2 h. The product was then purified with Zymo ssDNA/RNA purification kit or Amicon centrifugal filter.

XI. Generation of 5'-labeled RNA or modified RNA with PCT. DNA primer T2-F was 3'-labeled with 5-X-FAM-UTP or Biotin-11-UTP by terminal transferase (TdT). For the labeling reaction, 20 μM DNA primer T2-F was mixed with 100 μM 5-X-FAM-UTP or

Biotin-11-UTP, 0.25 mM $CoCl_2$, and 0.4 U/μL terminal transferase (TdT) in 1× TdT buffer, and incubated at 37 °C overnight. The labeled primer was then purified with the Qiaquick Nucleotide Removal Kit. For large-scaled preparation, DNA primer was also 3'-labeled with labeled ribonucleotides during solid-phase synthesis, for more efficient and homogeneous labeling. The 3'-labeled primer was then used to PCT amplify template T7 (SEQ ID NO:27) (Table 1), which contains an extra "A" in the n+1 site of the primer extension to pair with the 3'-end labeled UTP using PCT conditions and program as described above. The PCT product was then treated with TurboDNase to remove DNA in the PCT product. The 5'-FAM-labeled RNA/modified RNA product was assayed with 15% PAGE gel, and imaged with the Typhoon 9410 scanner using FAM channel. The 5'-Biotin-labeled RNA/modified RNA was then incubated with an excess amount of streptavidin (0.17 μg/μL) at 37 °C for 1 h, and assayed with 10% PAGE gel.

XII. Thrombin binding assay with 2'-F-RNA aptamer generated by PCT. Template T5 (SEQ ID NO:22), which encodes the 2'-F-C,U-RNA thrombin aptamer, was subjected to PCT as described above. The PCT product was then treated with TurboDNase to degrade DNA, and then purified with the Zymo ssDNA/RNA purification kit. The purified 2'-F-C,U-RNA was then folded in 1x binding buffer (20 mM HEPES, pH 7.5, supplemented with 150 mM NaCl, 6 mM KCl, and 2 mM $MgCl_2$) by heating at 75 °C for 5 min, and rapidly cooling on ice. The folded 2'-F-C,U-RNA was then incubated with 10 μM human α–thrombin at room temperature for 2 h, and the binding product was analyzed with 8% native PAGE gel.

XIII. Separation and purification of PCT products (Figure 13). PCT was carried out as described above with one DNA primer labeled with 3'-biotinylated rNTP (in this case, Biotin-11-UTP) and one regular DNA primer. The PCT product was then incu-bated with magnetic streptavidin C1 beads at 37 °C for 2 h. The beads were then washed 6 times with BWBS buffer (10 mM Tris•HCl pH 7.4, 1 M NaCl, 0.1% Tween20, 1 mM EDTA), and resuspended in 1× TurboDNase buffer and TurboDNase, and incubated at 37 °C for 2 h to degrade DNA and release the first RNA or modified RNA oligonucleotide which is not biotinylated. The beads were washed 6 times with BWBS buffer. To release the second RNA or modified RNA product, which is biotinylated, the beads were suspended in 98% formamide supplemented with 10 mM EDTA (pH 8.0), and heated to 90 °C for 10 min. The two RNA/modified RNA products were then analyzed by 18% denaturing PAGE gel containing 8 M urea.

*Example 10*

*Utility and Advantages*

In one embodiment, an advantage of the present disclosure and methods is the exponential production of orders of magnitude more RNA or modified RNA than is available by conventional transcription. For example, the inventors have demonstrated the ability of PCT reaction to exponentially synthesize large quantities of RNA and 2'-F-modified RNA, and at a fraction of the cost of conventional synthesis. As would be known to a skilled artisan, other modifications, besides 2'-F, may also be incorporated by the PCT reaction, and the disclosure herein is in no way limited to 2'-F-modified RNA. In one embodiment, unlike conventional RNA transcription, PCT has no sequence constraints and it facilitates purification and 5'-labeling.

SFM4-3 polymerase is thermostable, and thus the RNA/modified RNA synthesis can be carried out at a much higher temperature than that used in conventional transcription, which allows efficient melting of secondary structures in the template or product, thus allows for the transcription of templates that are difficult or impossible to otherwise transcribe, and will significantly decrease sequence bias of transcription. Thermostability of SFM4-3 also allows the PCR amplification of R/DNA, and via PCT, the exponential production of large quantities of RNA or modified RNA from only the requisite triphosphates and from small quantities of a DNA template.

PCT is more efficient and general than conventional transcription and can produce large amounts of any RNA or modified RNA oligonucleotide at a fraction of the cost of chemical synthesis, and would be useful in quick and cheap exploration of the activity of multiple oligonucleotides. The RNA/modified RNA products have no sequence limitation, and 5'-end can be labeled with a desired tag. While the DNA of the DNA-RNA chimeras initially produced by PCT was removed in the PCT reaction, its presence might prove useful for different applications, including purification or as the 'sticky bridge' of oligonucleotide assemblies. While the current implementation of PCT requires the production of two different oligonucleotides per DNA template, this is not a disadvantage in many applications, for example when two or more oligonucleotides are desired, such as when evaluating siRNAs or candidate aptamers.

In one embodiment, the PCT reaction is optimized for palindromic substrates. The broad substrate tolerance of SFM4-3 indicating that modifications other than 2'-F are likely to be accommodated. In one embodiment, the thermophilic DNA polymerase mutant SFM4-3 is further evolved to increase the efficiency of synthesizing RNA or modified RNA, as well

as for the ability to PCT amplify linger RNA or modified RNA. In one embodiment, PCT reaction conditions are also further optimized to further enhance yield and generality.

The various methods and techniques described above provide a number of ways to carry out the invention. Of course, it is to be understood that not necessarily all objectives or advantages described may be achieved in accordance with any particular embodiment described herein. Thus, for example, those skilled in the art will recognize that the methods can be performed in a manner that achieves or optimizes one advantage or group of advantages as taught herein without necessarily achieving other objectives or advantages as may be taught or suggested herein. A variety of advantageous and disadvantageous alternatives are mentioned herein. It is to be understood that some preferred embodiments specifically include one, another, or several advantageous features, while others specifically exclude one, another, or several disadvantageous features, while still others specifically mitigate a present disadvantageous feature by inclusion of one, another, or several advantageous features.

Furthermore, the skilled artisan will recognize the applicability of various features from different embodiments. Similarly, the various elements, features and steps discussed above, as well as other known equivalents for each such element, feature or step, can be mixed and matched by one of ordinary skill in this art to perform methods in accordance with principles described herein. Among the various elements, features, and steps, some will be specifically included and others specifically excluded in diverse embodiments.

Although the invention has been disclosed in the context of certain embodiments and examples, it will be understood by those skilled in the art that the embodiments of the invention extend beyond the specifically disclosed embodiments to other alternative embodiments and/or uses and modifications and equivalents thereof.

Many variations and alternative elements have been disclosed in embodiments of the present invention. Still further variations and alternate elements will be apparent to one of skill in the art. Among these variations, without limitation, are the selection of constituent modules for the inventive compositions, and the diseases and other clinical conditions that may be diagnosed, prognosed or treated therewith. Various embodiments of the invention can specifically include or exclude any of these variations or elements.

In some embodiments, the numbers expressing quantities of ingredients, properties such as concentration, reaction conditions, temperatures, and so forth, used to describe and claim certain embodiments of the invention are to be understood as being modified in some instances by the term “about.” Accordingly, in some embodiments, the numerical parameters

set forth in the written description and attached claims are approximations that can vary depending upon the desired properties sought to be obtained by a particular embodiment. In some embodiments, the numerical parameters should be construed in light of the number of reported significant digits and by applying ordinary rounding techniques. Notwithstanding that the numerical ranges and parameters setting forth the broad scope of some embodiments of the invention are approximations, the numerical values set forth in the specific examples are reported as precisely as practicable. The numerical values presented in some embodiments of the invention may contain certain errors necessarily resulting from the standard deviation found in their respective testing measurements.

In some embodiments, the terms "a," "an," and "the" and similar references used in the context of describing a particular embodiment of the invention (especially in the context of certain of the following claims) can be construed to cover both the singular and the plural. The recitation of ranges of values herein is merely intended to serve as a shorthand method of referring individually to each separate value falling within the range. Unless otherwise indicated herein, each individual value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g. "such as") provided with respect to certain embodiments herein is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention otherwise claimed. No language in the specification should be construed as indicating any non-claimed element essential to the practice of the invention.

Groupings of alternative elements or embodiments of the invention disclosed herein are not to be construed as limitations. Each group member can be referred to and claimed individually or in any combination with other members of the group or other elements found herein. One or more members of a group can be included in, or deleted from, a group for reasons of convenience and/or patentability. When any such inclusion or deletion occurs, the specification is herein deemed to contain the group as modified thus fulfilling the written description of all Markush groups used in the appended claims.

Preferred embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations on those preferred embodiments will become apparent to those of ordinary skill in the art upon reading the foregoing description. It is contemplated that skilled artisans can employ such variations as appropriate, and the invention can be practiced otherwise than specifically described herein.

Accordingly, many embodiments of this invention include all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

Furthermore, numerous references have been made to patents and printed publications throughout this specification. Each of the above cited references and printed publications are herein individually incorporated by reference in their entirety.

In closing, it is to be understood that the embodiments of the invention disclosed herein are illustrative of the principles of the present invention. Other modifications that can be employed can be within the scope of the invention. Thus, by way of example, but not of limitation, alternative configurations of the present invention can be utilized in accordance with the teachings herein. Accordingly, embodiments of the present invention are not limited to that precisely as shown and described.

## CLAIMS

What is claimed is:

1. A method of generating a RNA, comprising:

providing a reaction mixture comprising a DNA template, a forward primer, a reverse primer, a SFM4-3 polymerase, and ribonucleotides; and

generating the RNA by thermocycling the reaction mixture.

2. The method of claim 1, wherein the method makes two different types of RNA sequences per DNA template.

3. The method of claim 1, wherein the sequence of the DNA template is palindromic.

4. The method of claim 1, wherein two RNA sequences are transcribed simultaneously by using a non-palindromic DNA template sequence.

5. The method of claim 1, wherein concentration of the DNA template in the reaction mixture is between 0.1 nM and 20 nM.

6. The method of claim 1, wherein the RNA is generated in an exponential manner.

7. The method of claim 1, wherein the ratio of the RNA generated to the DNA template used in the reaction is about $10^2$, $10^3$, $10^4$, $10^5$, or $10^6$ times.

8. The method of claim 1, wherein the method of generating a RNA comprises a method of transcribing RNA and/or a method of amplifying RNA.

9. The method of claim 1, wherein the ribonucleotides are modified ribonucleotides.

10. The method of claim 1, wherein the thermocyling step comprises multiple cycles of steps a.-c.:

a. denaturating the reaction mixture by heating to about 90°C–95°C;
b. annealing the reaction mixture by cooling to about 35°C–50°C; and
c. transcribing the RNA sequence by heating to 45°C–55°C.

11. The method of claim 1, wherein the RNA generated is a modified RNA.

12. The method of claim 1, wherein the RNA generated is a 2'-modified RNA.

13. The method of claim 1, wherein the RNA generated is a 2'-F modified RNA.

14. The method of claim 1, wherein the RNA generated is a DNA-RNA chimera.

15. The method of claim 14, wherein the DNA of the DNA-RNA chimera is removed by incubation with a DNase.

16. The method of claim 1, wherein the either or both of the primers are 3'-labeled with a ribonucleotide, which comprises phosphorylation modifications, attachment chemistry/linker modifications, biotinylation, fluorophores, dark quenchers, spacers, modified bases, phosphorothioate bonds modifications, and/or click chemistry modifications, to generate 5'-labeled RNA/modified RNA products after DNase digestion of the PCT products.

17. A method for generating a RNA or modified RNA at an elevated temperature, comprising:

contacting a reaction mixture comprising a DNA template, a primer, SFM4-3 polymerase, and ribonucleotides; and

generating the RNA or modified RNA at the elevated temperature or by putting the reaction mixture through repeated cycles of heating and cooling.

18. The method of claim 17, wherein the RNA generated is a modified RNA.

19. The method of claim 17, wherein the RNA generated is a 2'-modified RNA.

20. The method of claim 17, wherein the RNA generated is a 2'-F modified RNA.

21. The method of claim 17, wherein the method further comprises nucleotides, modified nucleotides, modified ribonucleotides, or combinations thereof.

22. The method of claim 17, wherein the elevated temperature is between about 45°C–55°C.

23. The method of claim 17, wherein the primer is 3'-labeled with a ribonucleotide, which comprise phosphorylation modifications, attachment chemistry/linker modifications, biotinylation, fluorophores, dark quenchers, spacers, modified bases, phosphorothioate bonds modifications, and/or click chemistry modifications, to generate 5'-labled RNA/modified RNA products after DNase digestion of the transcription products.

24. A kit for transcribing, synthesizing, and/or amplifying a RNA sequence comprising:

SFM4-3 polymerase; and

ribonucleotides.

25. The kit of claim 24, wherein the kit further comprises further comprises dNTPs, modified dNTPs, modified rNTPs, or combinations thereof.

26. The kit of claims 24-25, wherein the modified dNTPs or rNTPs comprise phosphorylation modifications, attachment chemistry/linker modifications, biotinylation, fluorophores, dark quenchers, spacers, modified bases, phosphorothioate bonds modifications, and/or click chemistry modifications.

27. The kit of claims 24-26, wherein the nucleotides are isotopically labeled with $^{13}C$, $^{15}N$ and/or D isotopes.

28. The kit of claims 24-27, wherein the nucleotides comprises a 2' modified nucleotide, and/or an isotopically labeled nucleotide.

29. The kit of claims 24-28, further comprising a buffer.

30. The kit of claim 29, wherein the buffer comprises $MgCl_2$, Triton X-100, BSA, and Taq DNA polymerase buffer.

31. The kit of any one of the claims 24-29, further comprising instructions for using the kit.

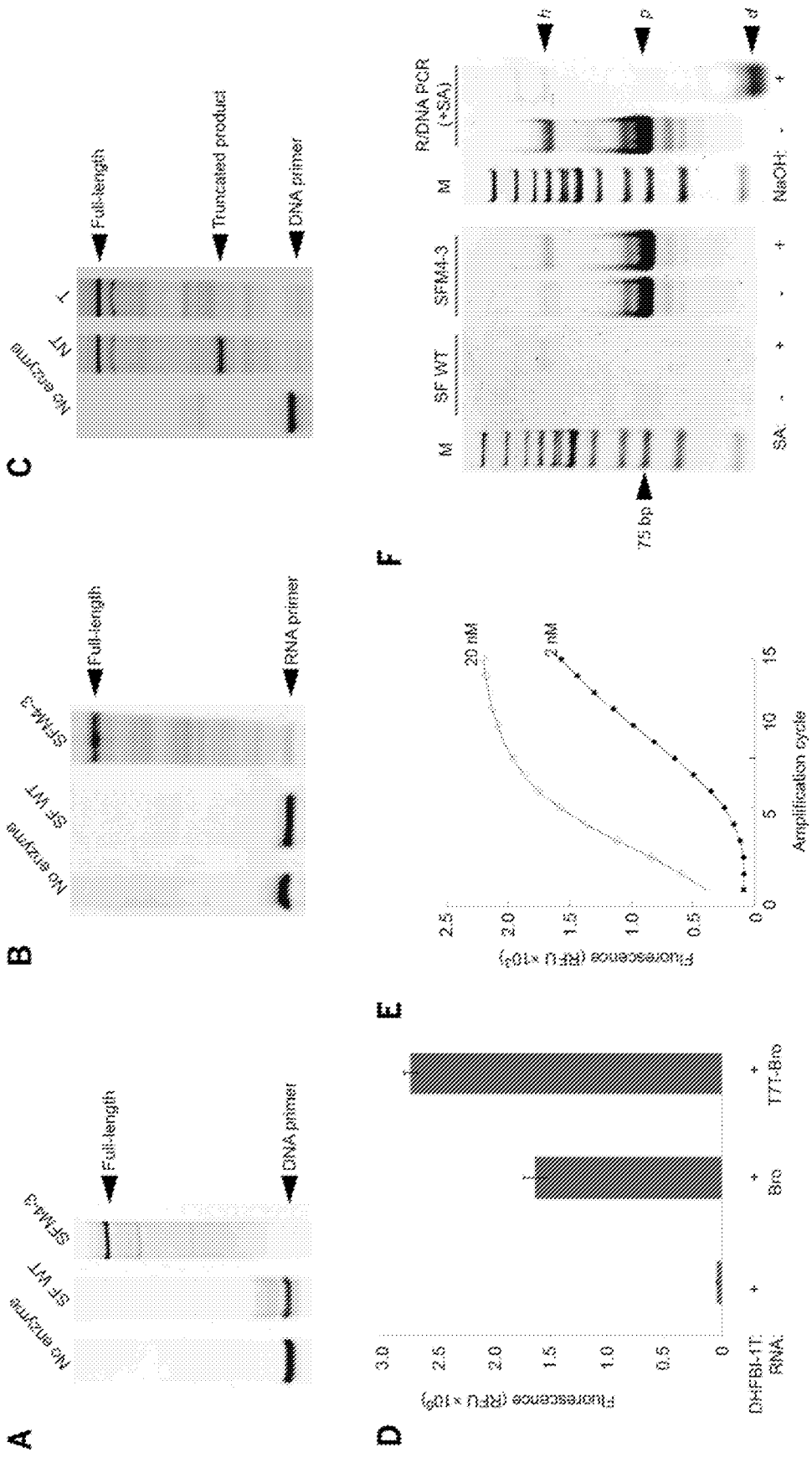
32. A kit comprising:

a thermostable mutant DNA polymerase for generating RNA; and

instructions for use, and

wherein the use comprise use of ribonucleotides as substrate for the polymerase to generate RNA.

Figure 1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RNA primer
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SFM4-3
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Truncated product
DNA primer
No enzyme
NT
T
D
Fluorescence (RFU × 10³)
DHFBI-1T RNA:
Bro
T7-Bro
E
20 nM
2 nM
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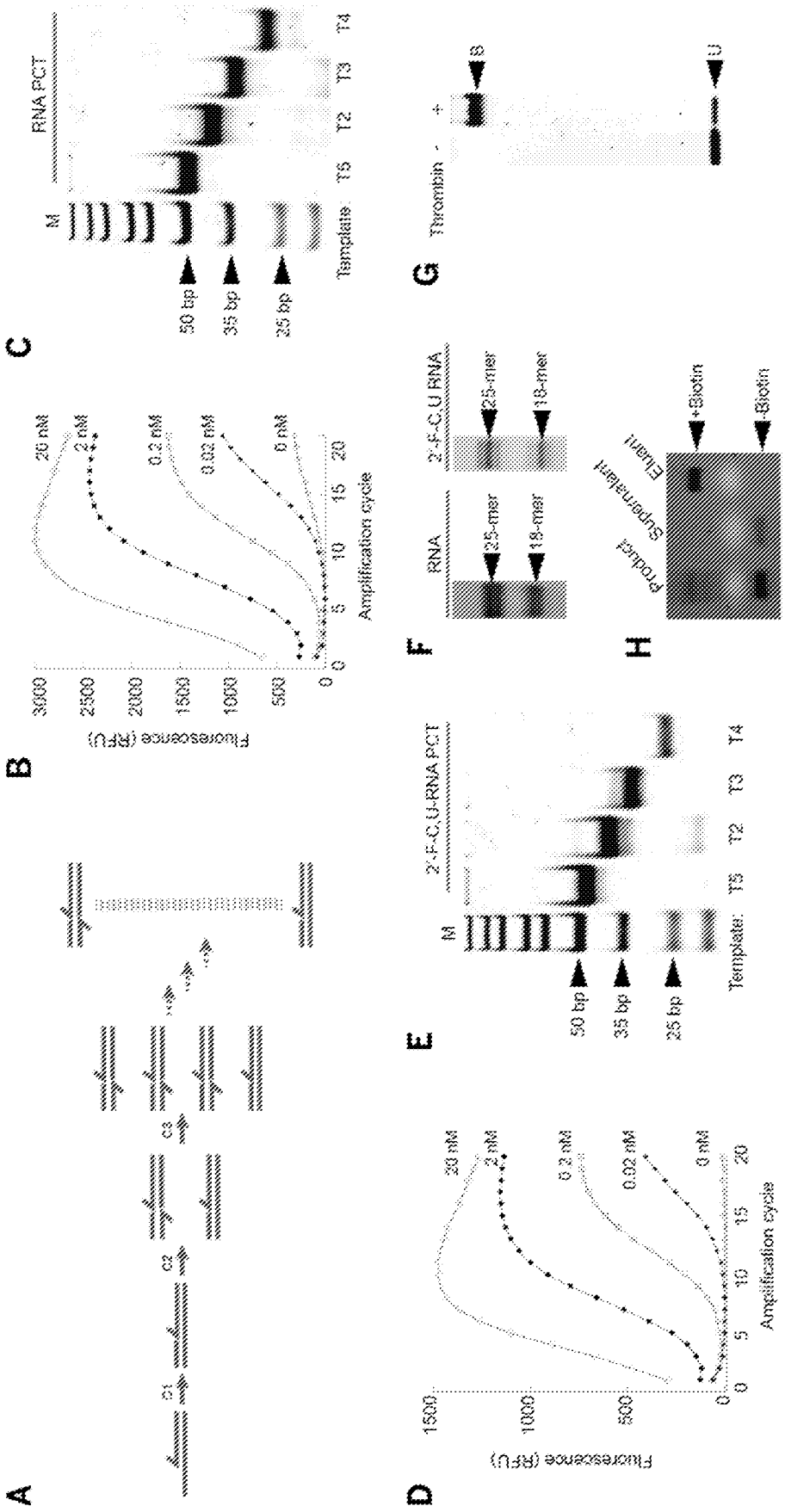
Figure 2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C3
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Fluorescence (RFU)
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M
Template
T5
T2
T3
T4
50 bp
35 bp
25 bp
D
2'-F-C,U-RNA PCT
E
F
RNA
2'-F-C,U RNA
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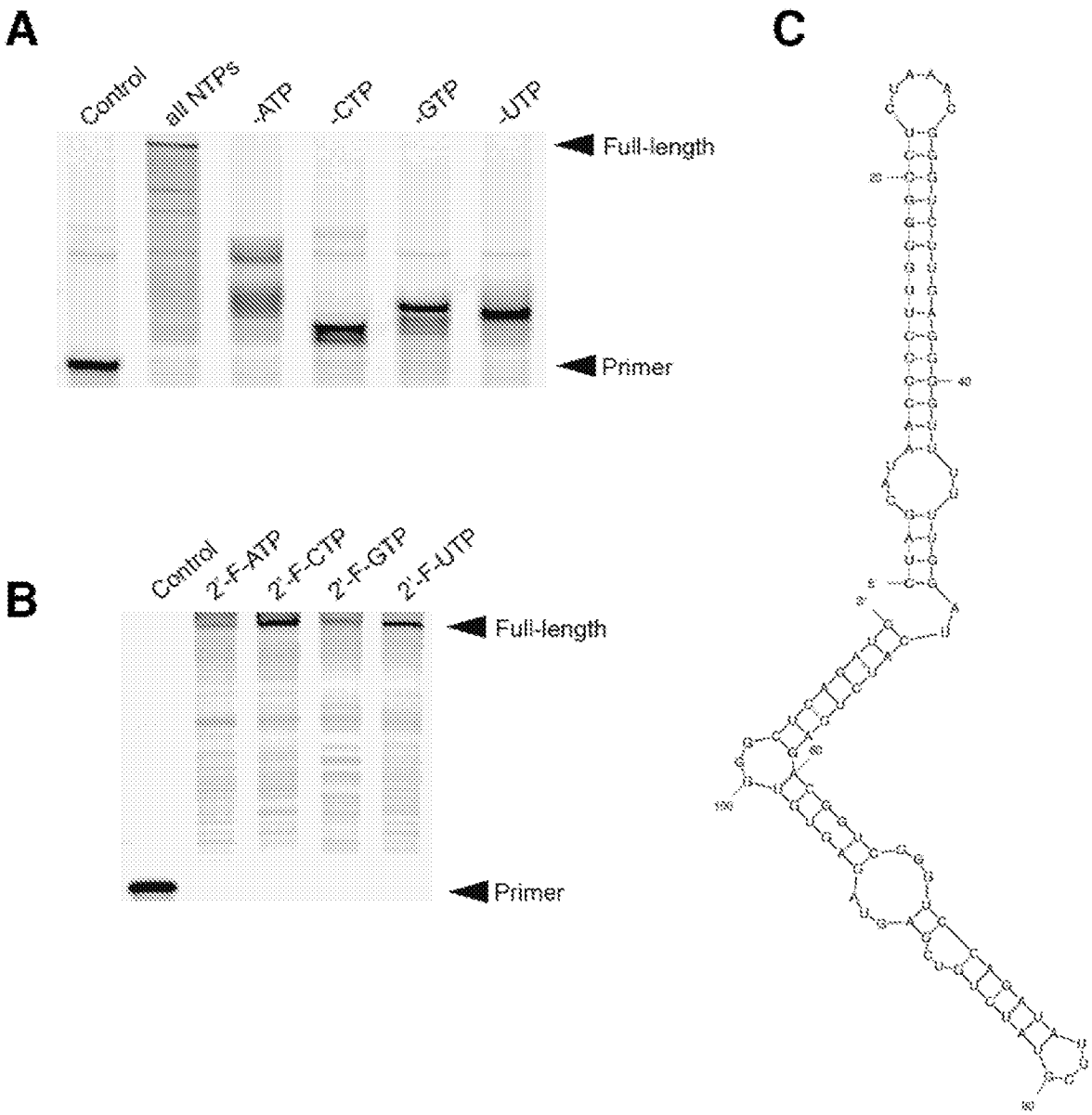
Thrombin
B
U
H
Product
Supernatant
Eluant
+Biotin
-Biotin

**Figure 3**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Control
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-CTP
-GTP
-UTP
Full-length
Primer
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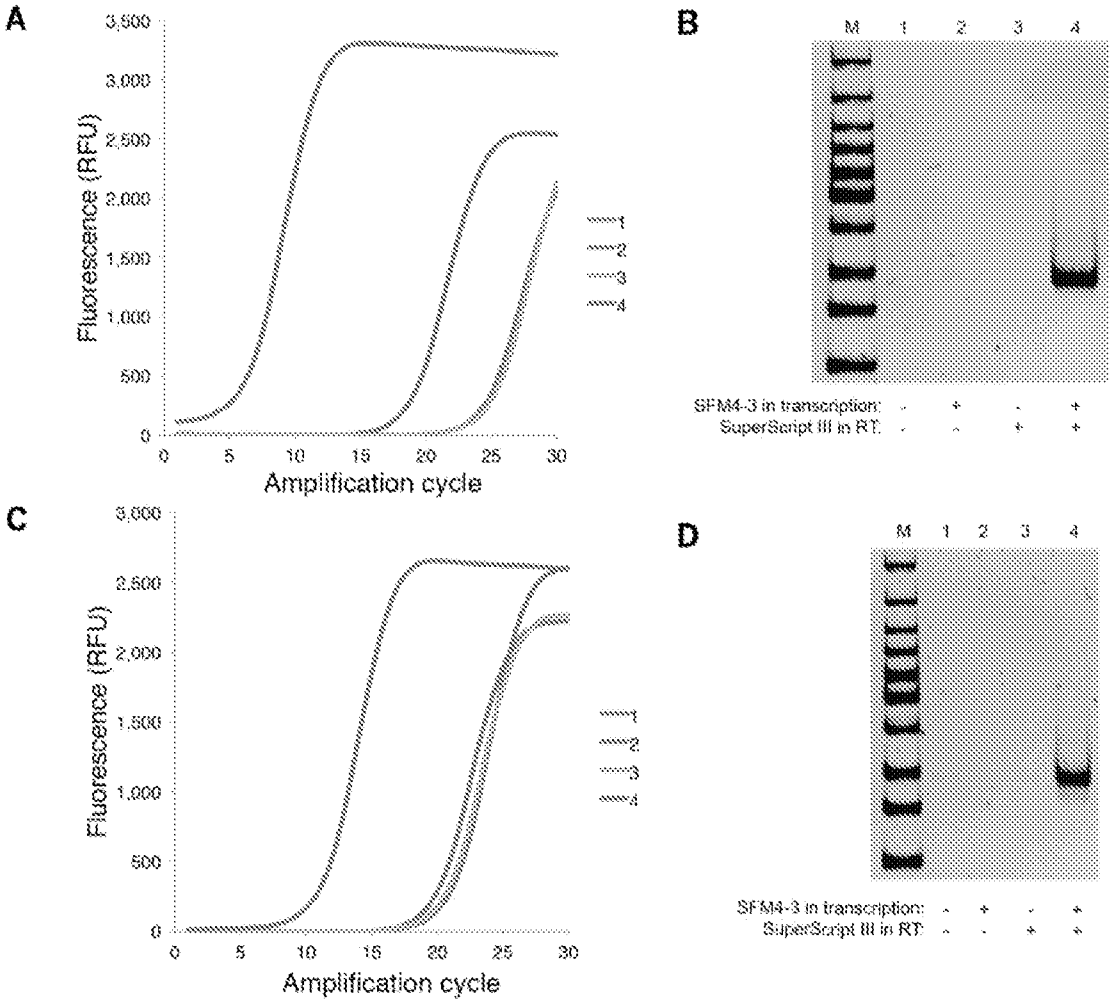
2'-F-GTP
2'-F-UTP
Full-length
Primer
C

**Figure 4**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Fluorescence (RFU)
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2,500
2,000
1,500
1,000
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Amplification cycle
1
2
3
4
B
M 1 2 3 4
SFM4-3 in transcription: - + - +
SuperScript III in RT: - - + +
C
Fluorescence (RFU)
3,000
2,500
2,000
1,500
1,000
500
0
0 5 10 15 20 25 30
Amplification cycle
1
2
3
4
D
M 1 2 3 4
SFM4-3 in transcription: - + - +
SuperScript III in RT: - - + +

Figure 5

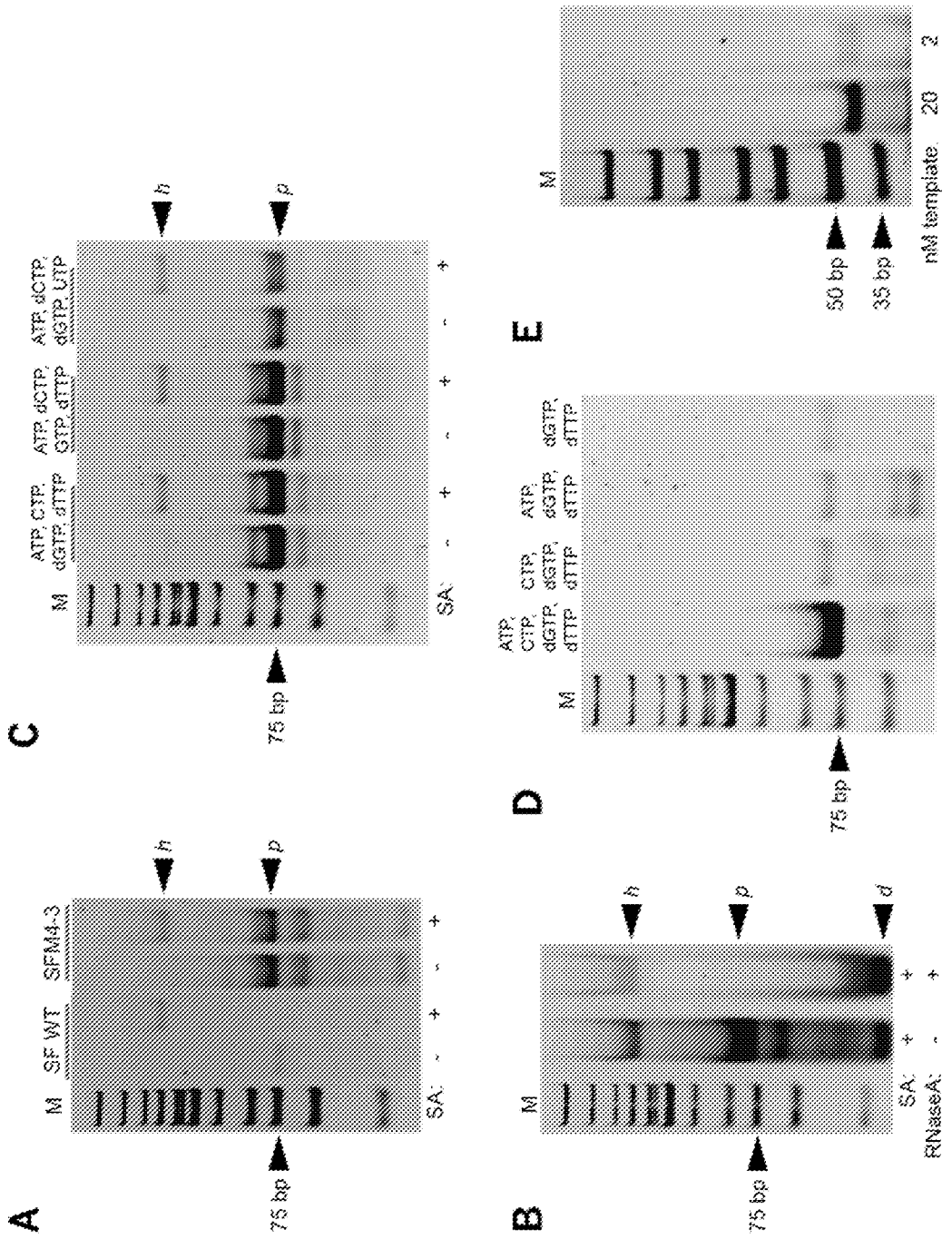

A
M
SF WT
SFM4-3
SA:
h
p
75 bp
B
M
SA:
RNaseA:
h
p
d
75 bp
C
M
ATP, CTP, dGTP, dTTP
ATP, dCTP, GTP, dTTP
ATP, dCTP, dGTP, UTP
SA:
h
p
75 bp
D
M
ATP, CTP, dGTP, dTTP
CTP, dGTP, dTTP
ATP, dGTP, dTTP
ATP, dGTP, dTTP
75 bp
E
M
50 bp
35 bp
nM template
20
2

Figure 6

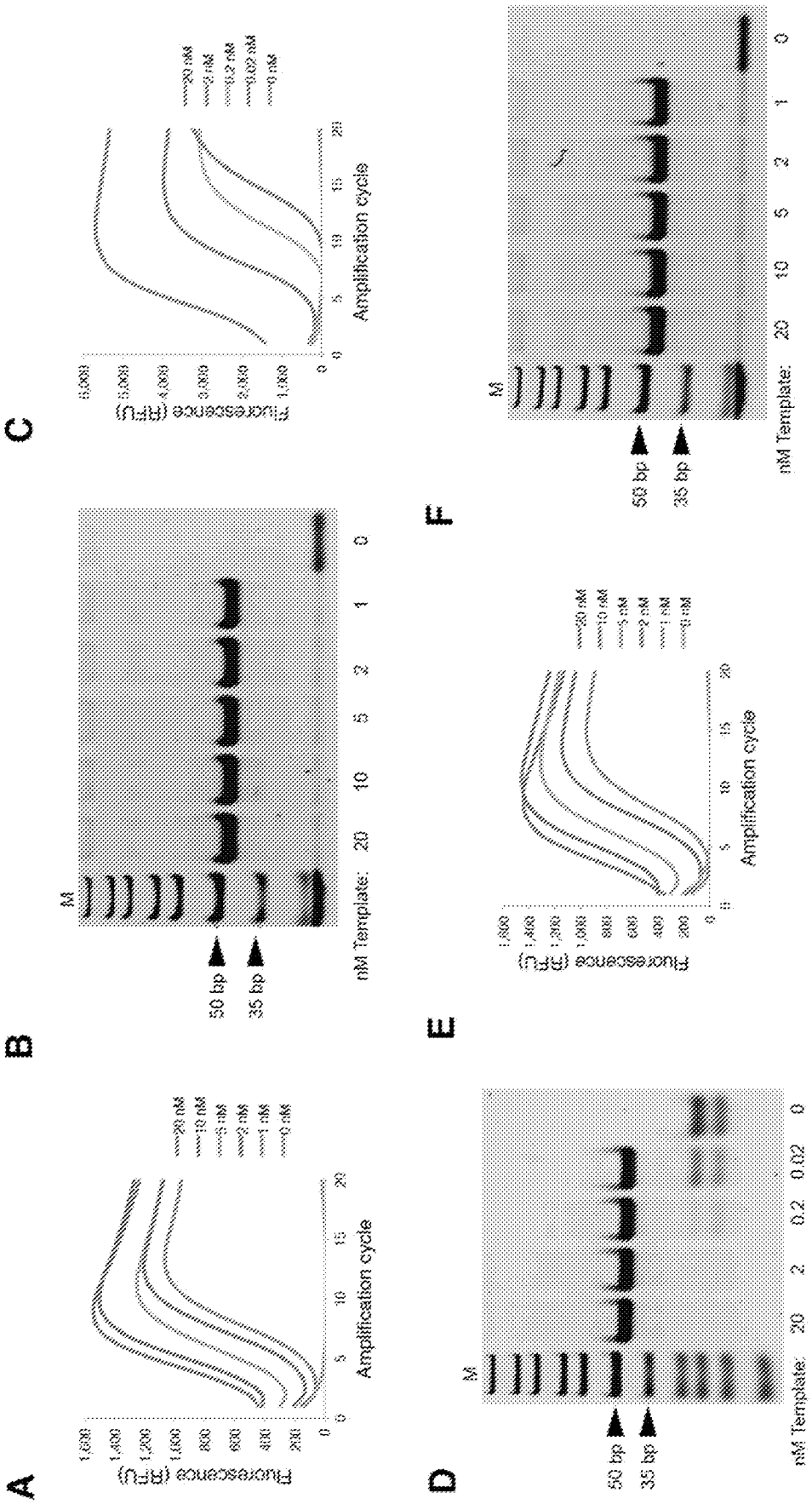

A
B
C
D
E
F
Fluorescence (RFU)
Amplification cycle
20 nM
10 nM
5 nM
2 nM
1 nM
0 nM
M
50 bp
35 bp
nM Template:
20
10
5
2
1
0
0.2
0.02

**Figure 7**

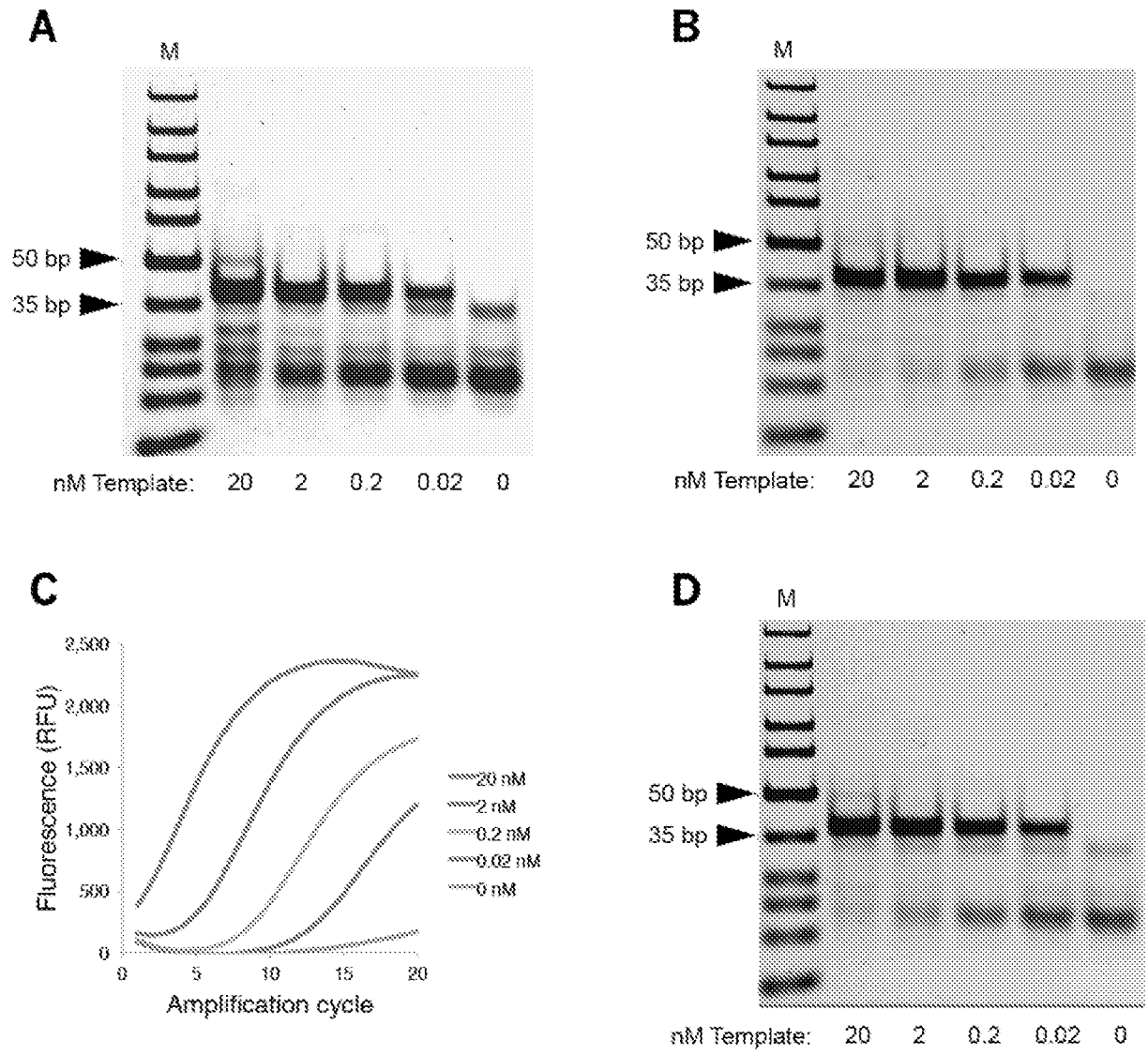

A
M
50 bp
35 bp
nM Template: 20 2 0.2 0.02 0
B
M
50 bp
35 bp
nM Template: 20 2 0.2 0.02 0
C
Fluorescence (RFU)
2,500
2,000
1,500
1,000
500
0
0 5 10 15 20
Amplification cycle
20 nM
2 nM
0.2 nM
0.02 nM
0 nM
D
M
50 bp
35 bp
nM Template: 20 2 0.2 0.02 0

**Figure 8**

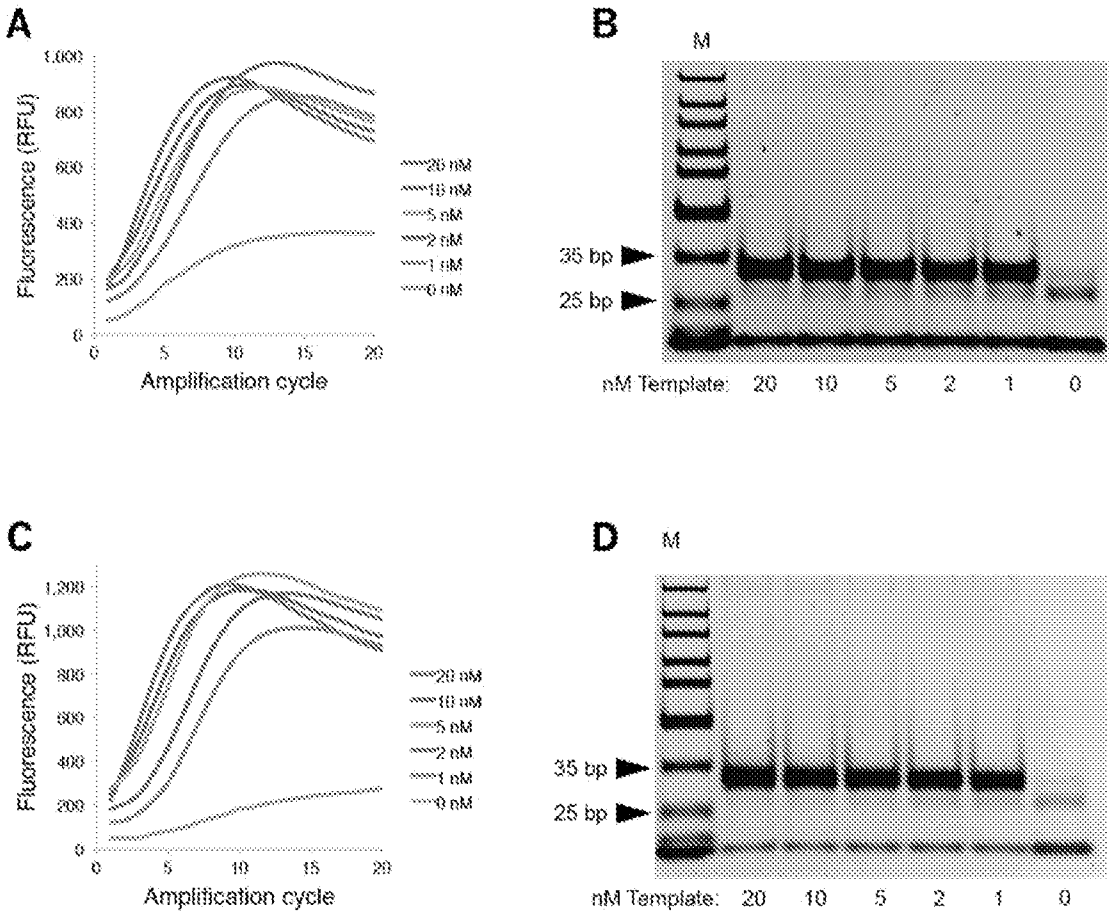
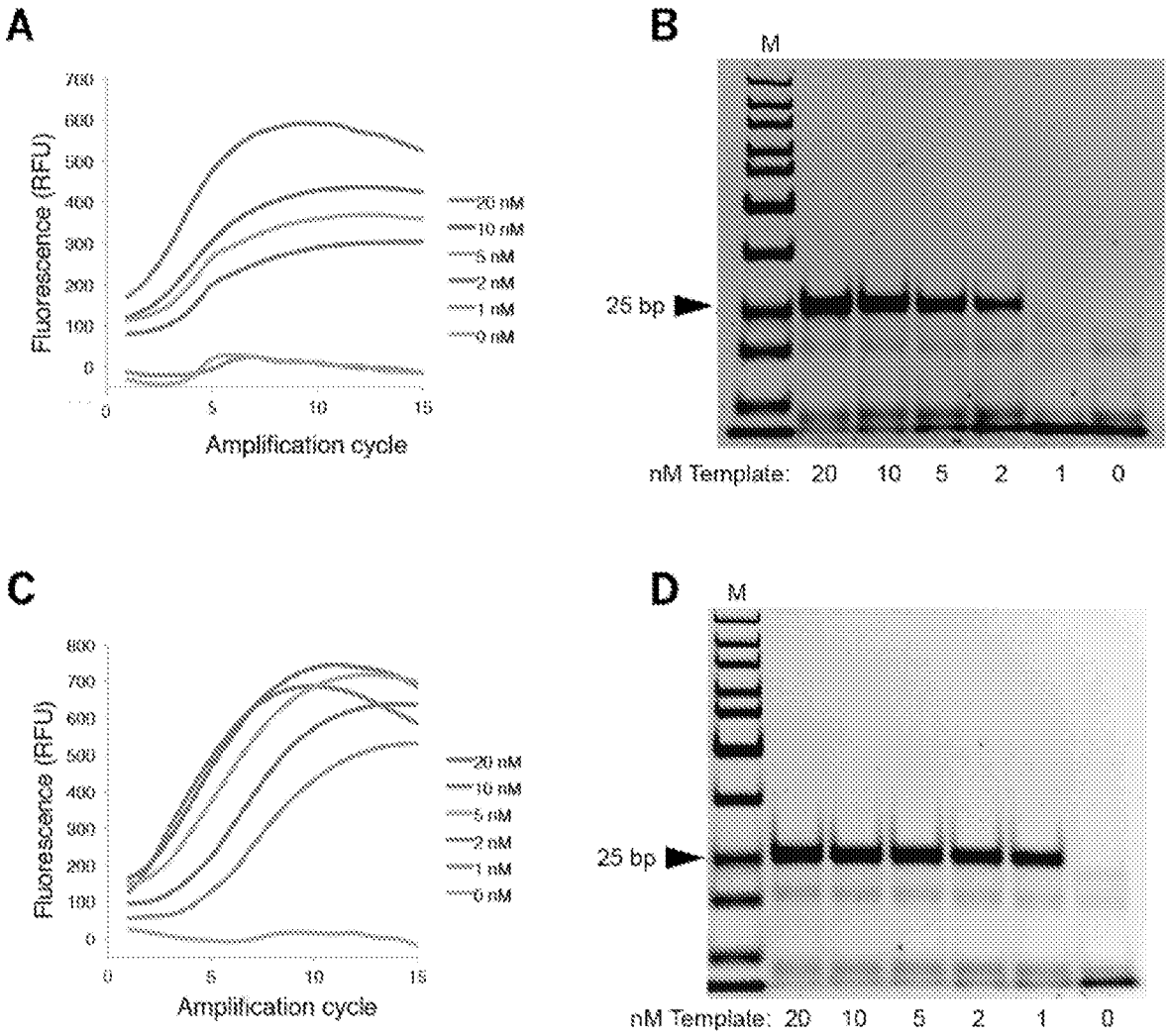

A
Fluorescence (RFU)
Amplification cycle
20 nM
10 nM
5 nM
2 nM
1 nM
0 nM
B
M
35 bp
25 bp
nM Template: 20 10 5 2 1 0
C
Fluorescence (RFU)
Amplification cycle
20 nM
10 nM
5 nM
2 nM
1 nM
0 nM
D
M
35 bp
25 bp
nM Template: 20 10 5 2 1 0

**Figure 9**


A
Fluorescence (RFU)
Amplification cycle
20 nM
10 nM
5 nM
2 nM
1 nM
0 nM
B
M
25 bp
nM Template: 20 10 5 2 1 0
C
Fluorescence (RFU)
Amplification cycle
20 nM
10 nM
5 nM
2 nM
1 nM
0 nM
D
M
25 bp
nM Template: 20 10 5 2 1 0

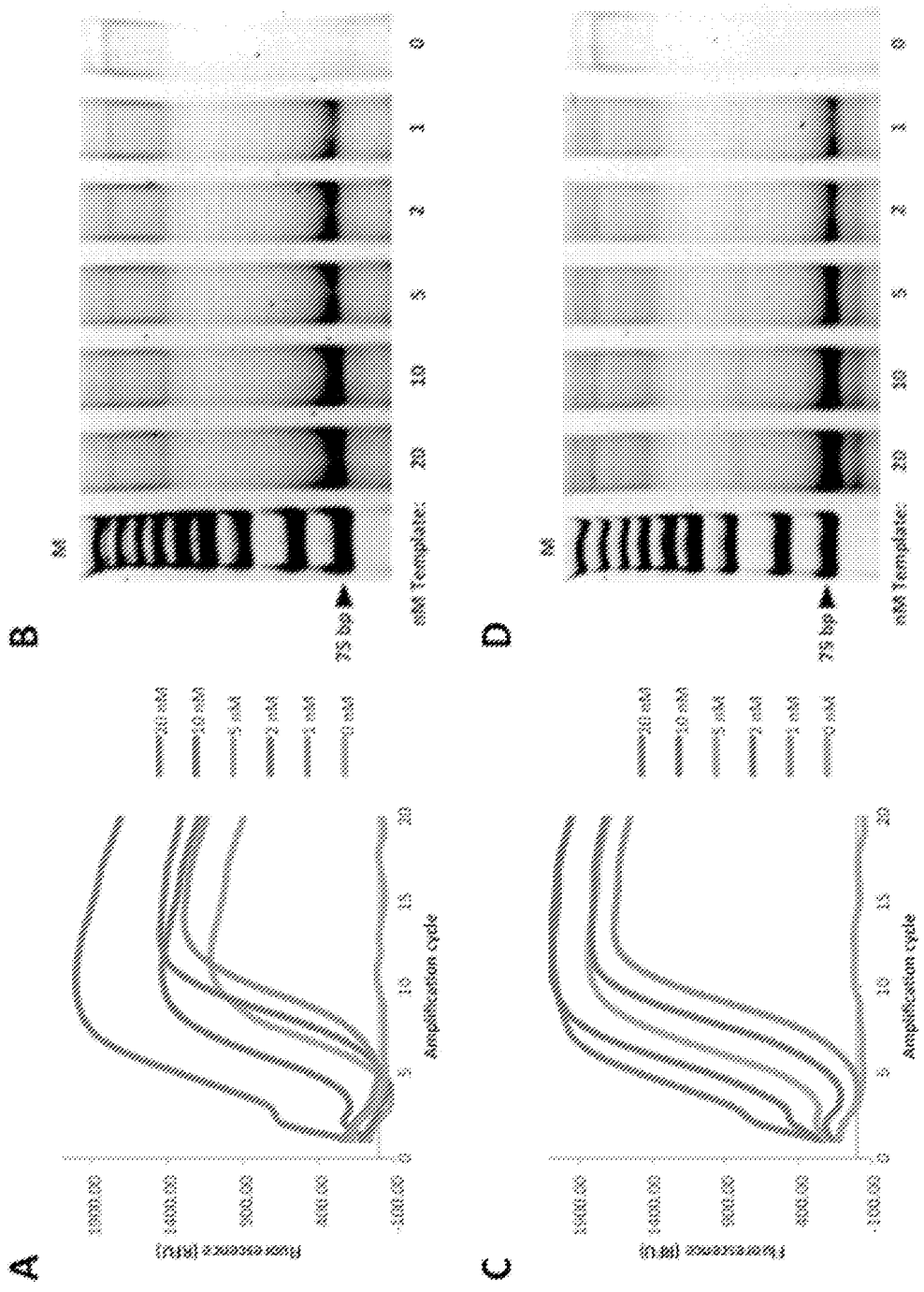



Figure 10

**Figure 11**

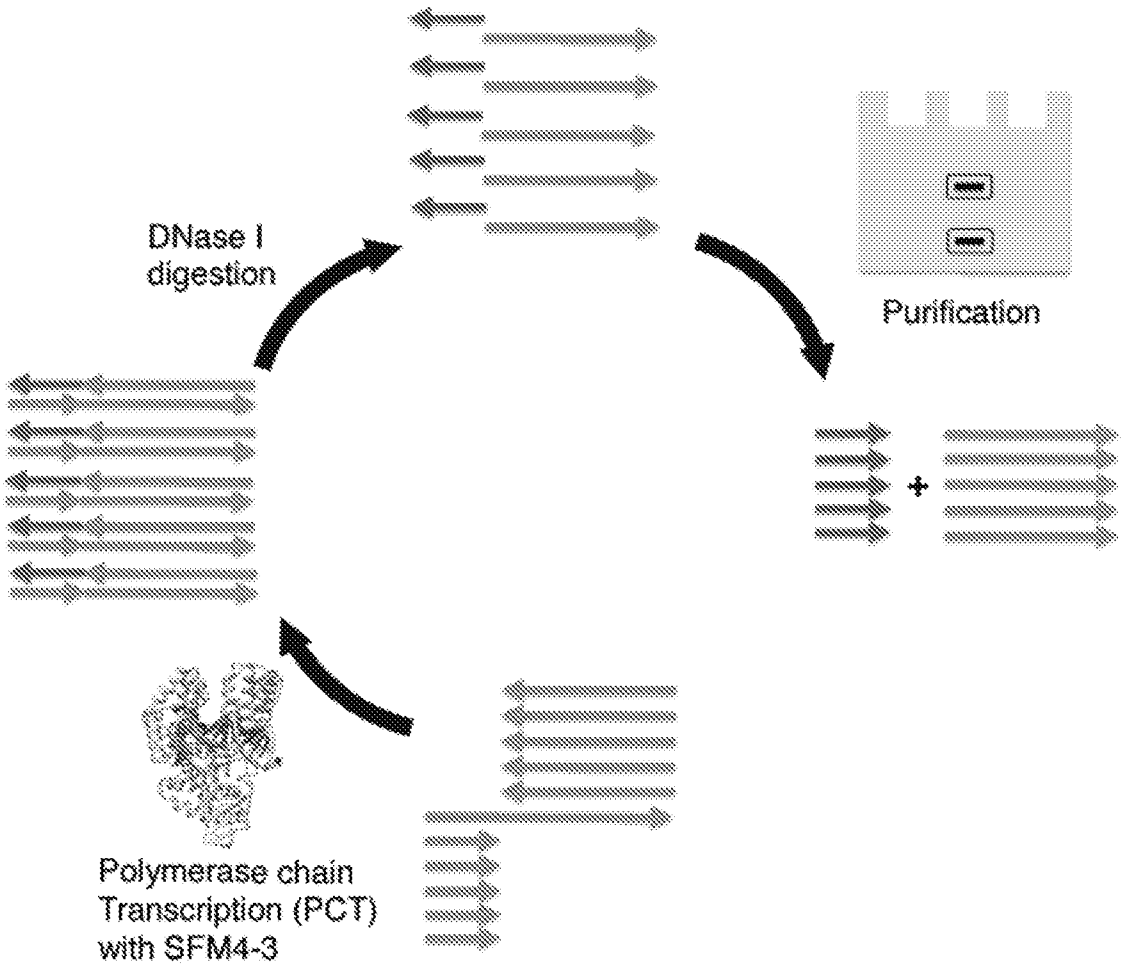
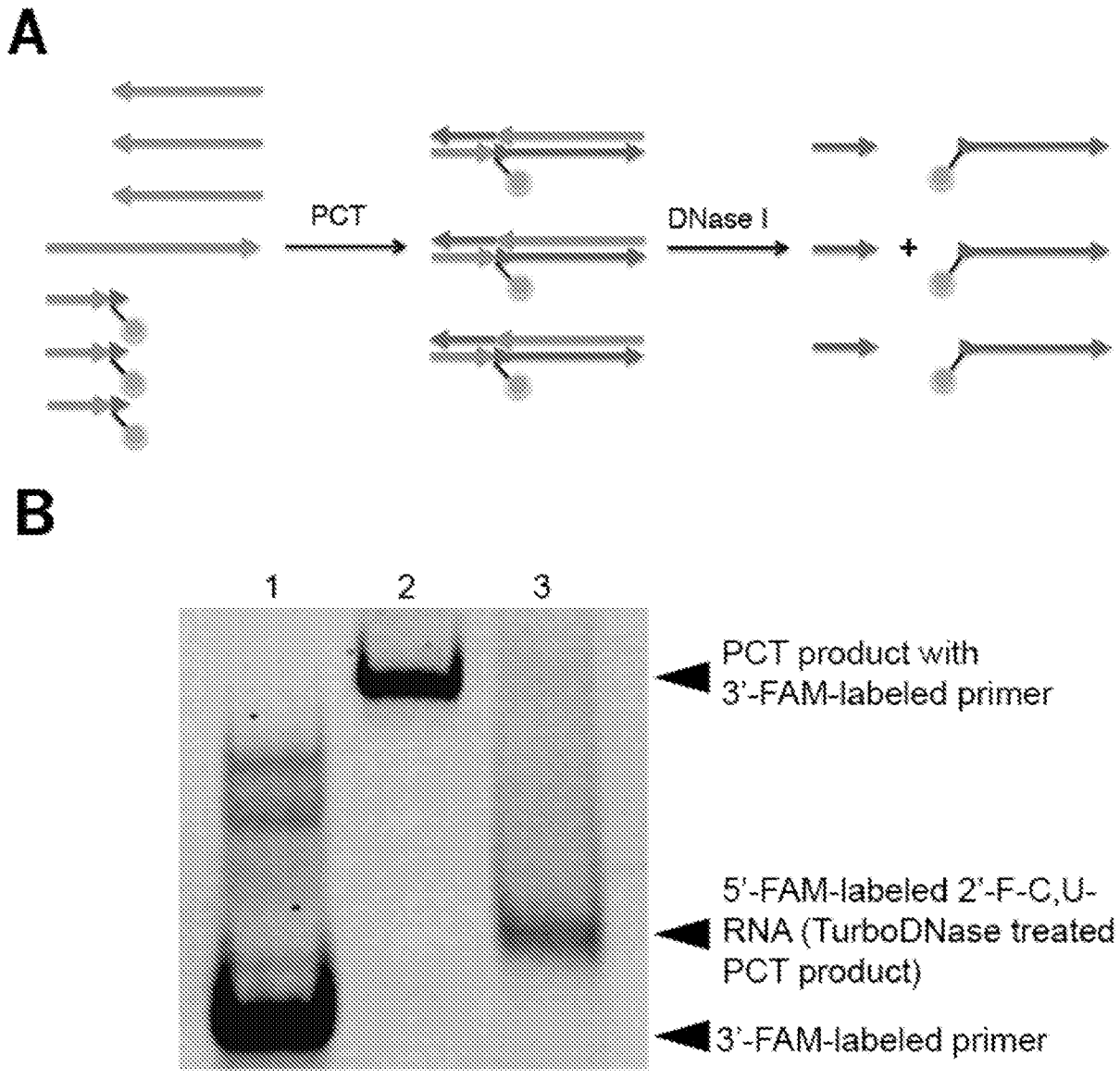

DNase I
digestion
Purification
Polymerase chain
Transcription (PCT)
with SFM4-3

**Figure 12**


A
PCT
DNase I
+
B
1
2
3
PCT product with
3'-FAM-labeled primer
5'-FAM-labeled 2'-F-C,U-
RNA (TurboDNase treated
PCT product)
3'-FAM-labeled primer

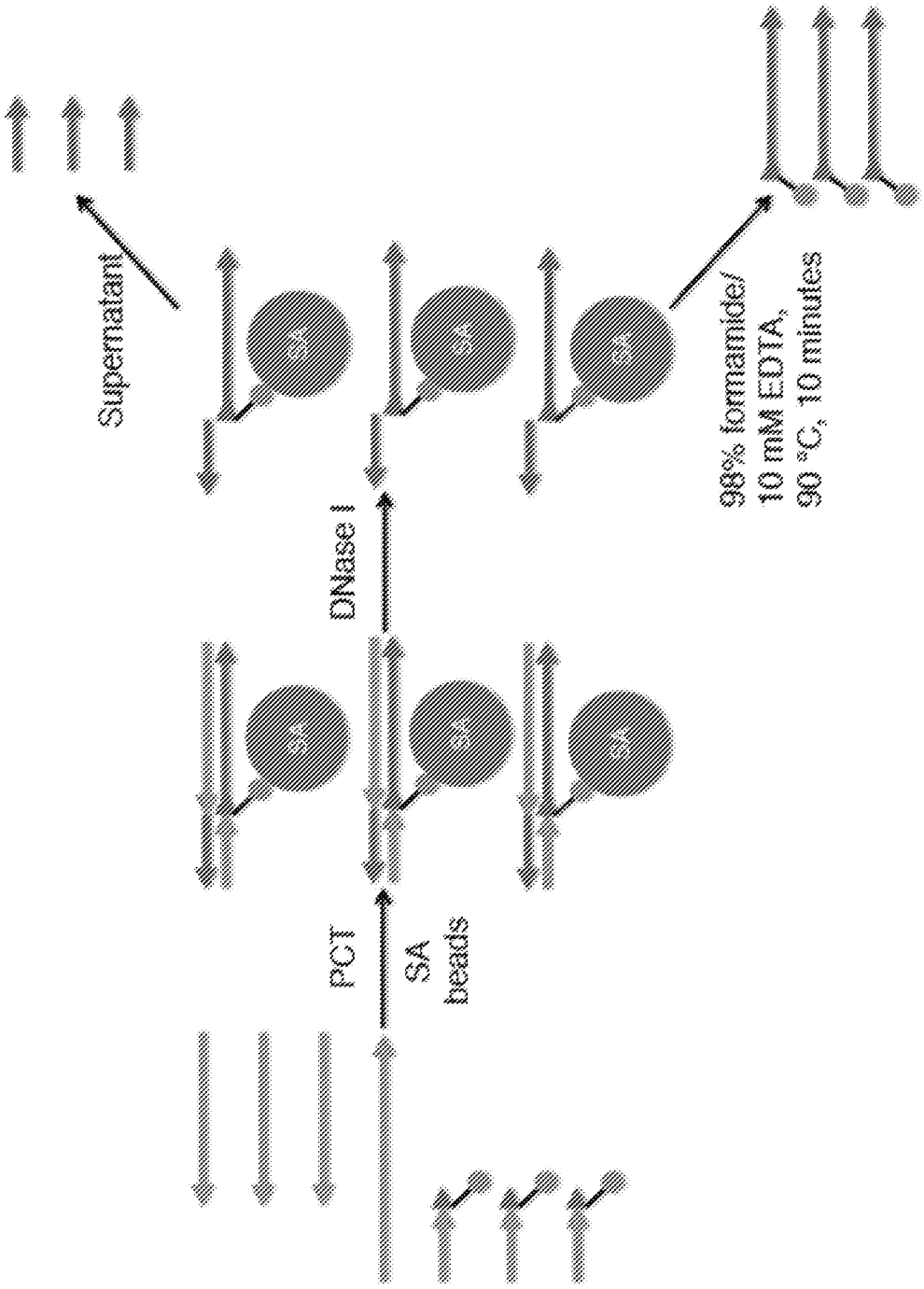

Supernatant
DNase I
PCT
SA beads
98% formamide/
10 mM EDTA,
90 °C, 10 minutes

Figure 13