Efficient Colorimetric Assay of RNA Polymerase Activity Using Inorganic Pyrophosphatase and Ammonium Molybdate

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Inorganic pyrophosphatases (PPases, EC 3.6.1.1) that can catalyze the hydrolysis of pyrophosphate (PP_i) into two orthophosphate (P_i) are often involved in many biochemical reactions to remove pyrophosphates formed during the reaction, which is driven by hydrolysis of nucleoside triphosphates.[1,2] The PPase activity can contribute to push the chemical equilibrium toward the biosynthesis of polyphosphoric acid such as DNA and RNA.[3] Therefore, continuous hydrolysis of pyrophosphate by pyrophophatases can enhance the yield of RNA or DNA synthesis during the nucleic acid polymerization. We have set out the experiments to monitor the activity of nucleic acids polymerase using the PPase, which can be applicable to a high-throughput screening of antiviral drugs against various viral RNA polymerases. Currently, activity assay of RNA polymerases is mainly based on the direct measurement of RNA polymerase's incorporation of radioactive ribonucleotides in the synthesizing nucleic acids. Such radioactive assays cannot be easily adapted to high throughput screening. In our studies, the amounts of inorganic phosphate are to be quantitatively measured with color-developing reagent after the RNA polymerase reaction, which is generated from hydrolysis of pyrophosphates by the PPase. This colorimetric coupled-enzyme system will facilitate measurement of RNA polymerase activity without need of cumbersome radioactivity assay.

Inorganic phosphate (P_i) can be detected and quantified by forming a unique blue-colored complex using molybdate and reducing agents.[4] Several colorimetric assays have been developed based on this reaction by using different reducing agents.[5-7] When ascorbic acid was used as the reducing agent, color intensity and sensitivity of phosphate-molybdate complex were greatly increased.[7-9] Due to its sensitivity and rapidness of the colorimetric assay for P_i quantification, there have been several reports that used this assay to monitor P_i from the phosphate-releasing enzyme reactions.[10-13] If the colorimetric detection of P_i is coupled with the activity of PPase, the P_i detection method could be developed to monitor the progress of biochemical synthesis of the nucleic acids. For example, Polymerase chain reaction (PCR) involves a continuous release of pyrophosphate by incorporating dNMP from dNTPs during the primer extension step. This pyrophosphate can be readily hydrolyzed by the PPase to produce two equivalents of orthophosphate. By monitoring the amount of inorganic phosphates released from pyrophosphate due to the catalytic activity of PPase, one can measure efficiency of DNA or RNA polymerases *in vitro*.

There have been several reports to describe nonradioactive assay of RNA polymerases. A colorimetric RNA polymerase assay has been described, which is based on incorporation of ATP or GTP with *p*-nitrophenyl moiety attached to γ-phosphate (PNP-NTP) during the RNA polymerase reaction.[14] PNP-pyrophosphate (PNP-PP_i) released during polymerization is simultaneously hydrolyzed by calf intestinal phosphatase to generate *p*-nitropheylate, which is monitored at 405 nm. This method has been applied to T7 bacteriophage RNA polymerase and brome mosaic viral replicase, and the incorporation of the modified nucleotides has been detected. However, the method used in the study did not prove efficient incorporation of the modified NTPs, which limits the application of the method to permissive systems. Other nonradioactive assay

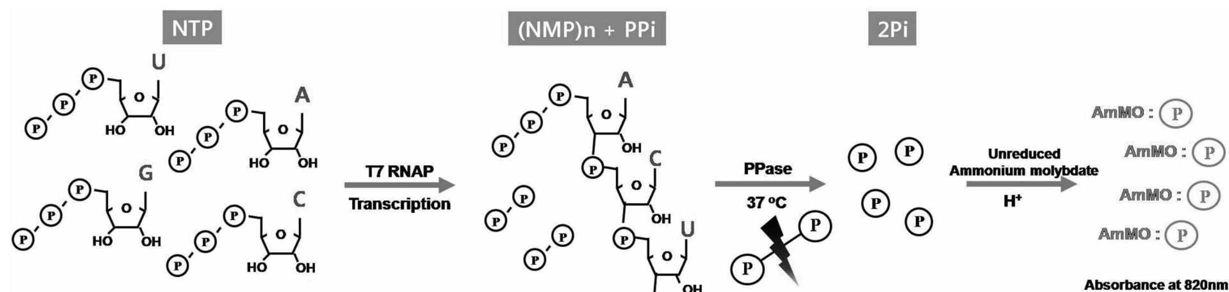

Figure 1. Reaction scheme for assay of RNA polymerase using the pyrophosphatase-coupled phosphate detection system.

method that utilizes a coupled-enzyme system has been reported to monitor the activity viral RNA-dependent RNA polymerases (RdRps).[15] The assay system is based on the chemiluminescent detection of PP_i, using ATP sulfurylase and firefly luciferase. The assay uses two enzymes for converting PP_i into chemiluminescent molecule: ATP sulfurylase converts PP_i into ATP and luciferase uses the converted ATP to produce chemiluminescence. Although the assay uses native NTPs instead of the modified nucleotides, use of ATPs to generate signal may obscure the condition of RNA polymerase reaction because ATPs are substrates for RNA polymerization as well.

As compared with the previous reports regarding the non-radioactive assay of RNA polymerase activity, in this paper we describe the development and evaluation of a simpler version of phosphate-based colorimetric assay to measure progress of nucleic acid polymerization by T7 bacteriophage RNA polymerase or *Taq* DNA polymerase using the PPase-coupled enzyme assay. Combined use of the PPase and colorimetric detection of inorganic phosphate could be applicable to assay many nucleic acid polymerases of interest to screen drug candidates at the early stage of drug screening *in vitro*. The assay was designed based on the reaction of robust yeast pyrophosphatase and the colorimetric P_i-detection method using the ammonium molybdate reagent (Fig. 1). Free PP_i generated as by-products during the nucleic acids polymerization is converted into two inorganic phosphates. The P_i produced by the yeast pyrophosphatase reaction accumulates during the polymerization reaction. At defined time points the accumulated P_i is quantified with the color-developing reagent.

As an initial experiment, PPase activity was determined by measuring the formation of inorganic phosphate at 37 °C. Addition of the acidified molybdate reagent efficiently quenched the pyrophosphatase reaction. In the quenched reaction deep blue color was developed due to the phosphomolybdate complex and its absorbance was measured at 820 nm in a microplate reader. The amount of inorganic phosphate was quantified using the phosphate standard curve, which was obtained by using serial dilutions of 1.00 mM NaH_2PO_4 standard solution. The released inorganic phosphates from increasing concentrations of pyrophosphate substrates incubated with the enzyme for 3 min were detected with the acidified molybdate reagent. All of these reactions were measured using a microplate spectrophotometer. Reactions containing 10 to 1000 μM PP_i and the PPase were incubated for 10 min at 37 °C to ensure linearity of P_i production in the reaction; less than 10% of the substrates should be hydrolyzed during the reaction. A control reaction was prepared by mixing all reagents, except PPase and the color development reagent, which gives the background P_i. As shown in Fig. 2, the amount of inorganic phosphate generated from pyrophosphate substrates in the pyrophosphatase reaction was readily detected with the color development reagent and increased as the substrate concentrations increase.

To investigate whether the PPase is useful in quantitative measurement of the activity of RNA polymerase during the polymerization reaction, we set out the *in vitro* transcription experiment to measure accumulation of RNA products as well as pyrophosphate during the RNA polymerization reaction by

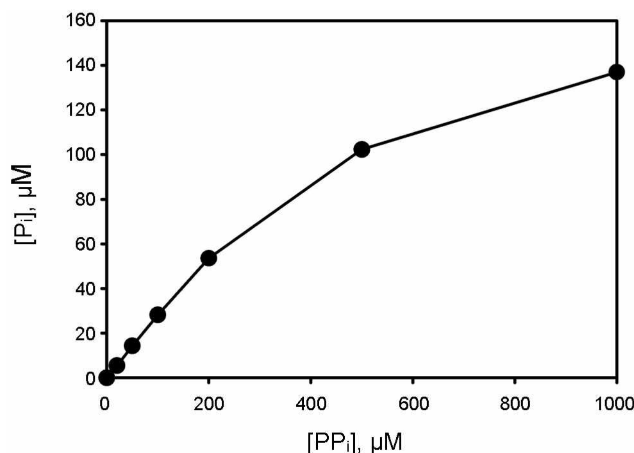

Figure 2. Standard curve for pyrophosphate and their colorimetric detection. The PPase was incubated with 10 to 1000 μM pyrophosphates at 37 °C and reactions were allowed to proceed for 10 min. The inorganic phosphate was detected with a phosphate detection reagent and its absorbance was measured at 820 nm. The concentration (μM) of inorganic phosphate was quantified using a phosphate standard curve.

T7 bacteriophage RNA polymerase. *In vitro* transcription using the T7 RNA polymerase was performed in duplicate at 37 °C. The denaturing polyacrylamide gel electrophoresis (10 % Urea-PAGE) analysis and the PPase-coupled assay were used for the measurement of synthesized RNA and accumulated PP_i in the reaction, respectively. The reaction mixture that was prepared by mixing the T7 RNA polymerase with the reaction buffer, template DNA, and NTPs was incubated at 37 °C for 3 hrs. At various time points of the reaction, 20 μL aliquot of the transcription mixture was quenched by adding 0.5 M EDTA. The quenched reaction was analyzed for synthesized RNAs with 10% Urea-PAGE (Fig. 3A). RNA products were accumulated during the *in vitro* transcription reaction with T7 RNA polymerase. Simultaneously, accumulation of pyrophosphate over time was also monitored by mixing the PPase with the aliquots from the RNA polymerase reaction at each time point. Inorganic phosphates were accumulated continuously as the RNA polymerase reaction progresses (Fig. 3B). These results suggest that the RNA polymerase reaction is readily assayed by measuring the amount of inorganic phosphate using the combined assay of PPase reaction and acidified ammonium molybdate reagent. Thus, the PPase-coupled reporting system legitimately reflects the activity of RNA polymerase *in vitro*.

In an effort to develop the PPase-coupled P_i detection system into the high throughput assay for nucleic acid polymerase activity, we further carried out the PPase-coupled inorganic phosphate assay for T7 RNA polymerase in the presence or absence of inhibitor, and *Taq* DNA polymerase. Reaction with each RNA and DNA polymerase produced inorganic phosphates over the time-course after the PPase-coupled reaction, which is readily shown in the 96-well plate with blue colored spots (Fig. 4). Inhibition of RNA polymerase activity caused by low level of $MgCl_2$, in which the reaction was supplemented with Mg-chelating EDTA, was also performed to test the validity of the assay system. As shown in Fig. 4,

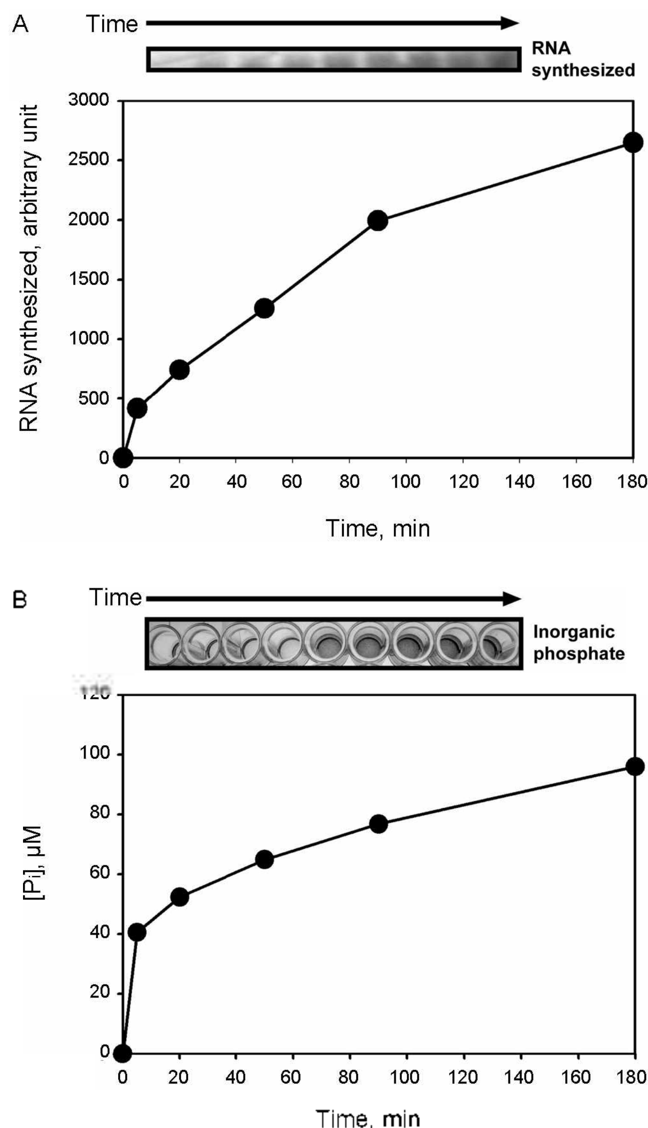

Figure 3. *In vitro* transcription for RNA synthesis and inorganic phosphate quantitation. *In vitro* transcription reaction was performed in duplicate for pyrophosphate hydrolysis reaction and polyacrylamide gel electrophoresis. (A) RNA products were analyzed by 10% Urea-PAGE and quantified by the Gel-Pro analyzer software. (B) At the same time points as in (A), the concentration of P_i (μM) accumulated in the PPase-coupled reaction was measured as stated in the text.

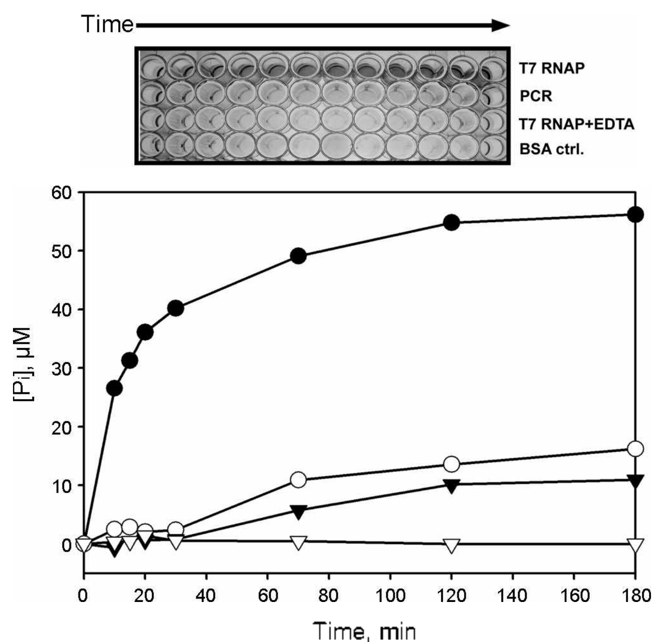

Figure 4. High-throughput assay of nucleic acid polymerases. In the 96-well plate, *in vitro* transcription reaction was performed and the P_i concentration (μM) was measured in a time-course manner. In the upper panel, 96-well plate is shown for monitoring absorbance of phosphomolybdate complex. 1st row is *in vitro* transcription reaction with T7 RNA polymerase (T7 RNAP); 2nd row is PCR with *Taq* DNAP (PCR); 3rd row is *in vitro* transcription reaction with low-$MgCl_2$ (T7 RNAP + EDTA); 4th row is *in vitro* transcription reaction with BSA (BSA control). In the lower panel, The P_i concentration of each row was quantified and plotted. Closed circle (●) represents T7 RNAP; Open circle (○) represents DNA synthesis with PCR; Closed triangle (▼) represents T7 RNAP with low-$MgCl_2$; Open triangle (▽) represents BSA control.

inhibited activity of T7 RNA polymerase with EDTA was readily reported by less accumulation of P_i, compared with uninhibited RNA polymerase. Taken together, the current PPase-coupled inorganic phosphate detection system can be applicable for activity assay of various nucleic acid polymerases, such as viral DNA polymerases and RNA-dependent RNA polymerases, which is crucial at the first stage of antiviral drug screening.

Experimental Section

Enzymes. T7 bacteriophage RNA polymerase was purified from the *Escherichia coli* BL21/pAR1219 cell line (kindly provided by Dr. Smita S. Patel, Robert Wood Johnson Medical School, NJ, USA) using the established procedures as described previously.[16] The purified T7 RNA polymerase shown as single band of 98 kDa in SDS-PAGE was found to be > 95% pure by Coomassie staining. The purified T7 RNA polymerase was stored at −80 °C in a storage buffer consisting of Na_3PO_4 (20 mM) pH 7.7, Na_3-EDTA (1 mM), dithiothreitol (1 mM), NaCl (100 mM), and (50%, v/v) glycerol.[17] The protein concentration was determined by measuring the absorbance at 280 nm in 8 M urea using the molar extinction coefficient of 1.4×10^5 $M^{-1}cm^{-1}$.[16] Yeast pyrophosphatase was purchased from Sigma as lyophilized powder form, and the sock enzyme was prepared by mixing with the same storage buffer as T7 RNA polymerase.

Pyrophosphate hydrolysis reaction. The 0.14 μM (10 μg/mL) of yeast PPase was mixed in 96 well with 30 μL of the reaction buffer (50 mM TrisHCl, pH 7.5, 5 mM $MgCl_2$, 10 ~ 1000 μM inorganic pyrophosphate). The reaction was started by the addition of enzyme. The amount of phosphate present in the reaction was determined by adding 70 μL of phosphate detection reagent (1.5% (w/v) ascorbic acid, 1% (w/v) ammonium molybdate, 0.42 M sulfuric acid) and incubated for 1 hr at 37 °C.[8] A deep blue color was developed due to the unreduced phosphomolybdate complex and its absorbance was measured at 820 nm in a microplate reader.

***In vitro* transcription and DNA synthesis.** *In vitro* transcription reaction was performed by mixing 0.5 μM T7 RNA poly-

merase with reaction buffer (40 mM Tris-HCl, 15 mM $MgCl_2$, 2 mM spermidine, 10 mM DTT), 2 mM NTPs (ATP, GTP, CTP, and UTP), and 0.2 μg/μL DNA templates. The reaction mixture was incubated at 37 °C for 3 hrs and the RNA products were analyzed by polyacrylamide gel electrophoresis (10% Urea-PAGE). Amount of RNA synthesized in the reaction was quantified by measuring the RNA band intensity using the Gel-Pro analyzer software. For pyrophosphate hydrolysis reaction, 20 μL aliquot of the RNA products which was obtained at each time point was mixed with 0.14 μM PPase and 10× reaction buffer (500 mM, Tris-HCl, pH 7.5, 50 mM $MgCl_2$) and incubated for 30 min at 37 °C. Measurement of pyrophosphate hydrolysis activity was performed as above. DNA synthesis reaction was performed with conventional PCR reaction. PCR was carried out by incubating 0.2 μg/μL of DNA template, 1 μM each of forward primer and reverse primer, 500 μM of dNTPs mixture, and 0.2 U *Taq* DNA polymerase (SunGenetics, Daejeon, Korea) in the PCR reaction buffer supplied by the vendor. PCR was performed for 33 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and 20 μL aliquot of the PCR mixture at designated time points was withdrawn and incubated with 0.14 μM PPase for 30 min at 37 °C. Subsequent colorimetric detection of inorganic phosphate in the reaction was performed as above.

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