Measurement of Nucleotide Triphosphate Sugar Transferase Activity via Generation of Pyrophosphate

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[Abstract] Nucleotide triphosphate (NTP) transferases (EC. 2. 7. 7. X) transfer a nucleoside monophosphate moiety from NTP to another substrate. NTP sugar transferases form a large member of the NTP transferase. There are many variations for the substrate combination of the NTP sugar transferases. It is important to measure the precise enzymatic activity of such NTP sugar transferases by a simple and efficient method. In our method, we measure pyrophosphate as a byproduct of nucleotide diphosphate (NDP)-sugar generation using the pyrophosphate assay kit. The kit reagents include two enzymes that convert pyrophosphate to phosphate, and then phosphorolyze chromogenic substrate to allow color development at 360 nm (see details below). Thus, the NDP-sugar formation can be simply traced as production of pyrophosphate, which is monitored by absorbance at 360 nm. This method is reliable and versatile for measurements of various pyrophosphate-producing enzymes that include NTP sugar transferases.

[Principle and overview] NTP transferases catalyze the reversible reaction as follows: NTP + sugar-1P <-> NDP-sugar + PPi

The enzyme reaction can be monitored as generation of inorganic pyrophosphate (PPi). The EnzChek Pyrophosphate Assay kit (Molecular Probes, Life Technologies, Carlsbad, CA) includes two enzymes and sufficient materials for color development to quantitate pyrophosphate. The inorganic pyrophosphatase (component E in the kit) degrades pyrophosphate into phosphate. Purine nucleoside phosphorylase (PNP, component B) utilizes phosphate to cleave the colorgenic substrate 2-amino-6-mercapto-7-methylpurine ribonucleoside (MESG, component A) into ribose-1-phosphate and 2-amino-6-mercapto-7-methylpurine. The product 2-amino-6-mercapto-7-methylpurine has the absorption maximum at 360 nm. The component C is the dilution solution that includes minimal $MgCl_2$ sufficient for the inorganic pyrophosphatase. Thus, NTP transferase activity can be monitored at 360 nm as generation of a byproduct pyrophosphate.

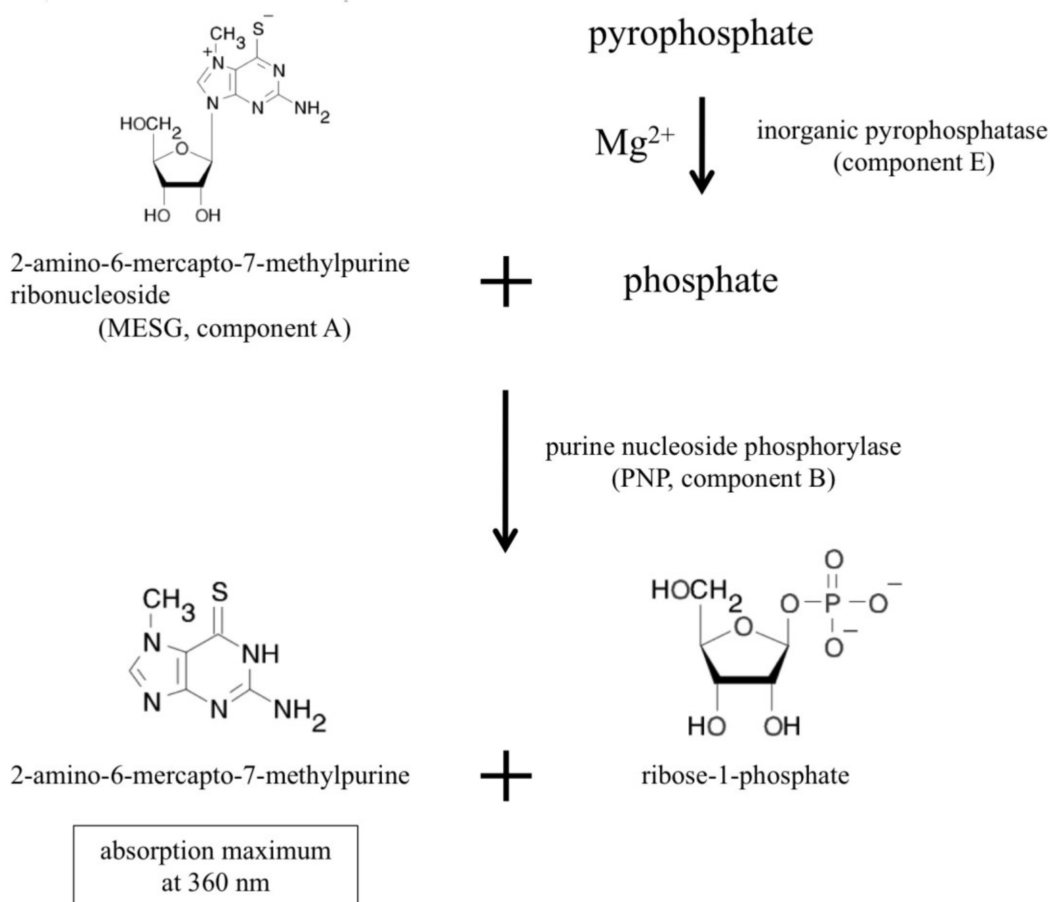

Figure 1. Chromogenic reactions in the assay kit

Typically, the nucleotidyl sugar transferase reactions have been measured by High Performance Liquid Chromatography (HPLC) (Kawano *et al.*, 2014). There are several advantages and disadvantages in HPLC method and our enzymatic method (O: advantage, X: disadvantage).

{HPLC method}

O) Can measure the reaction in both directions (NDP-sugar formation and degradation)

O) Can measure with a small amount of the sample protein

X) Can not follow the real-time reaction

X) The peaks of substrates and products must be separated in the chromatogram

{Pyrophosphate assay method}

O) Can observe the reaction in real-time

O) Easy to use many kinds of substrates because of the simple detection at 360 nm

O) Can be used in other pyrophosphate or phosphate generating reactions such as adenylate cyclase, diguanylate cyclase (Enomoto *et al.*, 2014), and DNA polymerase

X) Can not measure the pyrophosphate-consuming direction of the reversible reaction of NDP-sugar pyrophosphorylase. Km and kcat for NDP-sugar and pyrophosphate are not obtained by the pyrophosphate assay method but by HPLC method.

X) Need certain amount of the experimental protein. The maximum activity that the kit reagents allow corresponds theoretically to the rate of color development for the positive control is 2 x 10^{-2} U in our case. The instruction states the minimum detection of 5 x 10^{-5} U. Of course, the minimum activity we can measure may depend on the background activity due to contaminants in the substrates or in the sample preparation.

Materials and Reagents

1. EnzChek Pyrophosphate Assay kit (Molecular Probes, catalog number: E-6645)
    a. The contents of the components A~E are shown below.
    b. 2-amino-6-mercapto-7-methylpurine ribonucleoside (MESG)
    c. Purine nucleoside phosphorylase (PNP)
    d. 20x reaction buffer: 1.0 mM Tris-HCl, 20 mM $MgCl_2$ (pH 7.5)
    e. Pyrophosphate standard solution: 50 mM $Na_4P_2O_7$
    f. Inorganic pyrophosphatase
2. 1 M $MgCl_2$
3. 100 mM substrates (our examples in Table 1)
4. Enzyme preparation (with or without imidazole, see note 5 below)

Equipment

1. Block incubator (ASTEC, model: BI-516S)
2. Spectrophotometer (Shimadzu, model: UV-2600)
3. Electronic cooling and heating cuvette holder (Shimadzu, model: S-1700)
4. Quarts cuvette with a lid

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Table 1. An example of substrate reagents

Substrate	Company	Catalog number
Adenosine triphosphate (ATP)	Sigma-Aldrich	A-2383
Guanosine triphosphate (GTP)	Sigma-Aldrich	G-8877
Cytidine triphosphate (CTP)	Sigma-Aldrich	C-1506
Uridine triphosphate (UTP)	Sigma-Aldrich	U-6625
Deoxythymidine triphosphate (dTTP)	Sigma-Aldrich	T-0251
Glucose-1-phosphate (Glc-1P)	Sigma-Aldrich	G-9380
N-acetylglucosamine-1-phosphate (GlcNAc-1P)	Sigma-Aldrich	A-2142
Galactose-1-phosphate (Gal-1P)	Sigma-Aldrich	G-0380
Mannose-1-phosphate (Man-1P)	Santa Cruz Biotechnology	SC-284868

Procedure

A. Measurement of enzyme activity

1. Set up the spectrophotometer and electronic cooling and heating cuvette holder. Hold a cuvette in the holder to keep the temperature (usually 37 °C).
2. Prepare the assay kit solutions as written in the product manual and keep them on ice. The instruction states that component A should be used within 4 h when melted.
3. Prepare reaction mixtures according to Table 2.
4. Preincubate the mixture at 22 °C for 20 min to react the contaminated pyrophosphate and phosphate in the block incubator. We usually incubate the mixture for another 10 min at 37 °C (the measuring temperature) to warm up the mixture. The original instruction recommends the preincubation for 10 min at 22 °C.
5. Put the reaction mixture into the cuvette in the spectrophotometer and record the absorbance at 360 nm in a time-scan mode for 5 ~ 10 min. Usually, the linear time course of the color development was obtained often initial lag time of 10 ~ 20 sec. Therefore, it may be sufficient to record time points every 30 ~ 60 sec.

B. Data analysis

1. Measure the rate of absorbance change (ΔA_{360}/sec).
2. Convert the reaction rate (ΔA_{360}/sec) to the velocity (molar concentration/sec) by the difference of final A_{360} between the positive control and the negative control 1 (see the

formula below).

$$V\ (M / sec) = \frac{\Delta A_{360}\ (cm^{-1}) / sec - \Delta A_{360}\ (cm^{-1}) / sec\ (negative\ control)}{\varepsilon\ (M^{-1}\ cm^{-1})}$$

We experimentally determine the molar extinction coefficient of ~ 11,000 (M^{-1} cm^{-1}) at 360 nm every time based on the final absorption of the positive control as follows.

$$\varepsilon\ (M^{-1}\ cm^{-1}) = \frac{final\ A_{360}\ (cm^{-1},\ positive\ control) - final\ A_{360}\ (cm^{-1},\ negative\ control\ 1)}{pyrophosphate\ concentration\ (M,\ positive\ control)}$$

The kit provides standard solution of 50 mM pyrophosphate (component D). We obtained fairy reproducible value for ε in our hands although the instruction does not mention about the known ε value, the original literature (Upson *et al.*, 1996) states ε (M^{-1} cm^{-1}) = 11,000 at pH 7.6.

Table 2. Preparation of reaction mixtures

Purpose	Water (µL)	C* (µL)	A* (µL)	B* (µL)	E* (µL)	$MgCl_2$ (µL)	D* (µL)	Protein (µL)	(d)NTP (µL)	Sugar-1P (µL)	Total (µL)
Positive control	I 643	II 50	II 200	II 10	II 10	II 7	III 80				1000
Negative control 1	I 723	II 50	II 200	II 10	II 10	II 7					1000
Negative control 2	I 703	II 50	II 200	II 10	II 10	II 7			III 10	III 10	1000
Negative control 3	I 713-X	II 50	II 200	II 10	II 10	II 7		III X	III 10		1000
Negative control 4	I 713-X	II 50	II 200	II 10	II 10	II 7		III X		III 10	1000
Measurement	I 703-X	II 50	II 200	II 10	II 10	II 7		III X	III 10	IV 10	1000

The asterisk represent the kit components A~E. I~IV: The order of addition for preparation of reaction mixtures.

Representative data

Here are representative data of cyanobacterial UDP-glucose pyrophosphorylase expressed and purified from *E. coli* cells (Maeda *et al.*, 2014). The initial slope of the graph was used for calculation. We usually use enzymes at 50 ~ 200 nM, and substrates [(d)NTP and sugar-1P] at 50 µM ~ 2 mM.

Table 3. Preparation of reaction mixtures in our example

Purpose	Water (μL)	C (μL)	A (μL)	B (μL)	E (μL)	$MgCl_2$ (μL)	D (μL)	Protein (μL)	UTP (μL)	Glc-1P (μL)	Total (μL)
Positive control	I 643	II 50	II 200	II 10	II 10	II 7	III 80				1000
Negative control	I 683	II 50	II 200	II 10	II 10	II 7			III 20	III 20	1000
Measurement	I 563	II 50	II 200	II 10	II 10	II 7		III 120	III 20	IV 20	1000

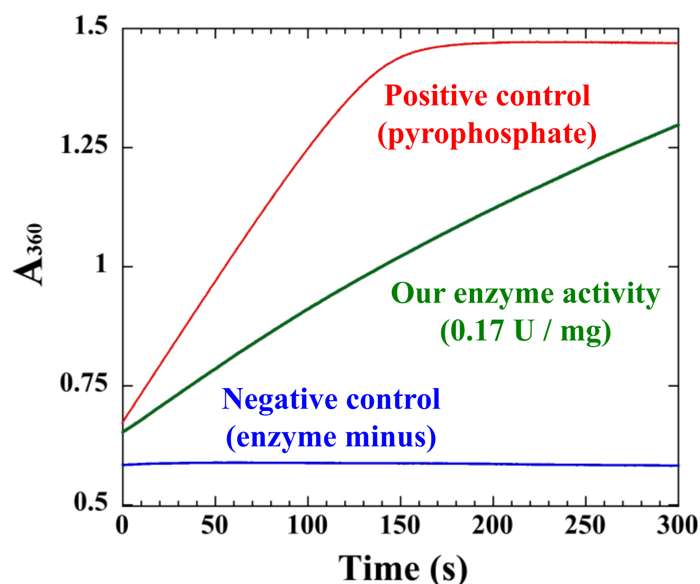

Figure 2. An example of measurements for polyhistidine-tagged cyanobacterial UDP-glucose pyrophosphorylase, which was purified by Ni-affinity chromatography

Notes

1. Before measurement, it is essential to check the reaction capacity of kit. Add 50~75 μM sodium pyrophosphate to the kit to confirm the maximum activity of pyrophosphorylase and purine nucleoside phosphorylase in the kit. We cannot measure NTP transferase activities higher than the maximum activity of the kit (Figure 2).
2. To check the quality of the assay kit, we must measure the positive control first.
3. Negative control 2 gives the level of contamination of phosphate and pyrophosphate. Negative control 3 and 4 give background enzyme activities to degrade (d)NTP and sugar-1P, respectively. Phosphate and pyrophosphate in the sample solutions or substrate reagents increase the background absorbance at 360 nm. We experienced some reagents such as mannose-1-phosphate contained too much phosphate. Such impurities may depend

on the lot of the reagent or product manufacturer.

4. A simple representative example for an enzyme sample is shown in Figure 2. The slope of the sample should be between the pyrophosphate positive control and the negative control. The initial slope of the graph was used for calculation according to the Data analysis.
5. The imidazole (up to final 100 mM in the assay mixture) has little effect on the assay kit activity. This means that the kit accepts usual preparations of His-tagged enzymes, which are (partially) purified by Ni-affinity chromatography.
6. Manganese ion (Mn^{2+}) inhibits the assay kit activity at final 5 mM or even lower concentration.
7. Reproducibility of the measurements is pretty high, though the maximum activity of the kit depends on the freshness of the kit.
8. According to the kit instructions, the reagents of the kit are stable for six months to one year at < -20 °C. Reconstituted MESG (component A) may be stored at < -20 °C for at least one month. Reconstituted purine nucleoside phosphorylase (component B) may be stored at 4 °C for at least one month. Diluted inorganic pyrophosphatase (component E) may be stored at 4 °C for at least one week.

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