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bio-protocol www.bio-protocol.org/e3705 Bio-protocol 10(15): e3705. DOI:10.21769/BioProtoc.3705

The ATPase Activity of *Escherichia coli* Expressed AAA$^+$-ATPase Protein

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[Abstract] ATPases are the enzymes that breakdown ATP to ADP and release inorganic phosphate (Pi). Here we provide a detailed protocol to determine the ATPase activity of a recombinant AAA$^+$-ATPase protein (GENERAL CONTROL NON-REPRESSIBLE-4 [GCN4]) by spectrophotometric absorption at 360 nm to measure the accumulated inorganic phosphate. In general, the substrate 2-amino-6-mercapto-7-methylpurine riboside (methylthioguanosine, a guanosine analog: MESG) is enzymatically converted in the presence of Pi by purine nucleoside phosphorylase (PNP) to ribose 1-phosphate and 2-amino-6-mercapto-7-methylpurine. The spectrophotometric shift in maximum absorbance at 330 nm for the MESG substrate and subsequent conversion product at 360 nm due to enzymatic conversion was measured. The GCN4-His-tagged recombinant protein was expressed in *Escherichia coli* BL21 cells and purified using Ni-NTA column. This purified protein was then used for the quantitation of Pi in solution or the continuous determination of Pi released due to the ATPase activity of GCN4, an AAA$^+$-ATPase protein conserved in many eukaryotes, which in plants regulates stomatal aperture during biotic and abiotic stress in plants.

Keywords: GCN4, ATPase, AAA$^+$-ATPase, MESG, Spectrophotometer

[Background] Adenosine triphosphatases (ATPases) are a class of enzymes that catalyze the breakdown of ATP into ADP and free inorganic phosphate (Pi). This breakdown and release of Pi generates energy used by enzymes to carry out the chemical reactions that require energy. This process is an integral part of all the kingdom of life. ABC transporters are the transmembrane proteins that move solute through the membrane by ATPase activity (Rees *et al.*, 2009). Some ATPases are cytoplasmic or membrane-associated proteins. AAA$^+$ proteins are the ATPases associated with diverse cellular activities such as protein degradation, membrane fusion, disassembly of protein complexes, microtubule dynamics, *etc.* (Snider *et al.*, 2008). We identified GENERAL CONTROL NON-REPRESSIBLE-4 (GCN4), an AAA$^+$-ATPase, as a novel player in regulating stomatal aperture and thus playing a role in plant innate immunity and drought tolerance (Kaundal *et al.*, 2017). Here we describe the method to prove its ATPase activity. The GCN4 as an ATPase enzyme converts ATP to ADP and Pi, and then the enzyme phosphoribosyl phosphorylase (PNP) converts the MESG substrate into the final substrate. The assay monitors the spectrophotometric shift of the substrate (MESG) in the presence of Pi from 330 to 360 nm. The conversion rate is directly correlated with the amount of Pi present in the solution and, that depends on the conversion of ATP to ADP by GCN4 ATPase activity. We reported the ATPase activity

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of a recombinant protein (GCN4-His) as absorbance (ABS) at 360 nm vs. protein concentration (Kaundal *et al.*, 2017).

Many techniques have been used to quantify the *in vitro* ATPase activity of purified proteins. Most of the techniques for quantification are based on the detection of free inorganic phosphate (Pi). The most common method uses the calorimeter substrate malachite green (Carter and Karl, 1982). Besides this, fluorescent and radioactive substrates are also used in various protocols to detect free phosphate in the ATPase reaction (Brune *et al.*, 1994; Shiue *et al.*, 2006). In this protocol, we used 2-amino-6-mercapto-7-methylpurine riboside (methylthioguanosine, a guanosine analog: MESG) as a substrate to detect the presence of free phosphate during ATPase activity (Webb, 1992). The purine nucleoside phosphorylase (PNP) enzyme in the presence of inorganic phosphate, which is released from ATP (an experimental substrate) upon hydrolysis to ADP by ATPase (experimental enzyme to be tested), converts MSEG (assay substrate) to ribose 1-phosphate and 2-amino-6-mercapto-7-methylpurine. This enzymatic conversion of MSEG results in a spectrophotometric shift in maximum absorbance from 330 nm for the MESG substrate to 360 nm for the product 2-amino-6-mercapto-7-methylpurine (Figure 1). The advantage of this protocol is that it does not require long incubation steps and the reaction can be incubated in the plate reader itself for the required duration. The protocol is based on the ENZcheck Phosphate Assay Kit. Most of the components for enzyme assay come with the kit, including potassium phosphate standard, so the reaction is very convenient to assemble. We optimized the protocol from 1 ml reaction to 300 µl reaction to carry out in a microtiter plate. This protocol describes the detailed assay for calculating the ATPase activity of a recombinant GCN4, an AAA$^+$-ATPase protein in U/ml, and specific activity. This protocol can also be used for the calculation of GTPase activity of a protein by using GTP as a substrate (Webb and Hunter, 1992).

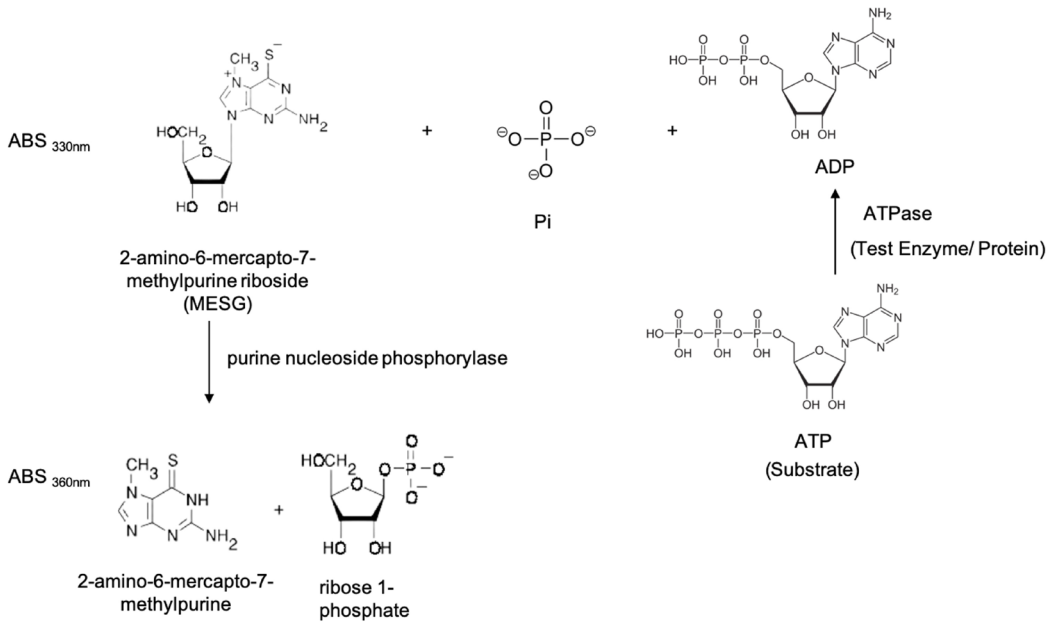

Figure 1. Enzymatic conversion of 2-amino-6-mercapto-7-methylpurine riboside (methylthioguanosine, a guanosine analog: MESG) into ribose 1-phosphate and 2-amino-6-

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mercapto-7-methylpurine by purine nucleoside phosphorylase (PNP) in the presence of inorganic phosphate released from ATP (substrate) by ATPase (enzyme to be tested)

Materials and Reagents

1. 96-well microtiter plate (BD Biosciences, catalog number: 353075)
2. EnzCheck Phosphate Assay Kit (Thermo Fisher, catalog number: E6646)
3. ATP (Sigma, catalog number: A2383)
4. ATPase (Sigma, catalog number: A7510)
5. Bio-Rad Protein Assay (Bio-Rad Laboratories, catalog number: 500-0006)
6. Bovine Serum Albumin (BSA) 50 mg/ml (Invitrogen™, catalog number: 15561020)
7. Tris base
8. Recombinant ATPase protein/enzyme you would like to test (we used GCN4-HisTag Fusion protein and referred to as test protein below)
9. 1 M Tris-Cl (pH-8.0) (see Recipes)

Equipment

1. Microtiter plate reader (Tecan, model: Infinite M200 Pro)
2. Water bath (Thermo Scientific, catalog number: TSGP02)
3. Vortex (FisherBrands, catalog number: 14-955-163)
4. Autoclave

Procedure

A. Estimation of protein concentration by Bradford assay (Bradford, 1976)
   1. Prepare a 100 µg/ml stock solution of BSA in water from BSA 50 mg/ml.
   2. Use a 96-well microtiter plate to prepare the reaction mix. Make triplicates for all reactions.
   3. Prepare 2.5, 5, 10, 20, and 50 µg of BSA standard in corresponding well as described in Table 1 below:

Table 1. Protein estimation reaction mix composition

Well	Concentration µg/ml	BSA (100 µg/ml) µl	Water µl	Bradford reagent µl
SD1	2.5	4	156	40
SD2	5	8	152	40
SD3	10	16	144	40
SD4	20	32	128	40
SD5	30	64	96	40
SD6	50	80	80	40

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4. Prepare a blank by adding 160 µl of water in a blank microtiter plate well.
5. Prepare test samples in a microtiter plate well by making several dilutions of purified recombinant ATPase protein/enzyme with water to make a total of 160 µl volume.
6. Add 40 µl of Bradford reagent to each microtiter plate well, mix and incubate for 5 min at room temperature.
7. Read absorbance (ABS) at 595 nm on a microtiter plate reader.
8. If the spectrophotometer does not have software to plot the standard curve, then plot the standard curve in Excel by plotting known BSA concentrations on X-axis and ABS_{595} on Y-axis. Obtain trendline and use the equation for the trendline and the ABS_{595} of the unknown to resolve the unknown concentration. The representative data are shown in Figure 2.

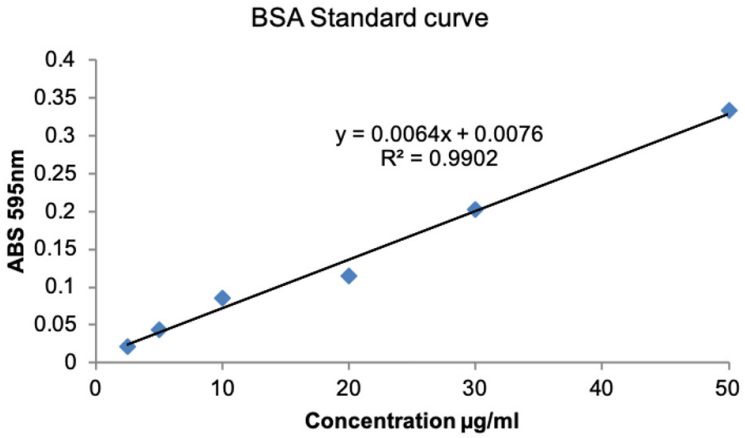

Figure 2. BSA standard curve

B. ATPase assay

Reagent preparation

Prepare stock solutions supplied with the EnzCheck Phosphate Assay Kit to perform the assay.

1. Prepare a 1 mM stock solution of the MESG substrate by adding 20 ml of dH_2O directly to the bottle (Component A). Mix extensively to dissolve MESG completely (maybe ~10 min).
   Note: Do not heat to dissolve. Because MESG is near its saturation point, a small amount of solid may remain, even after extensive mixing. Immediately after dissolving the MESG substrate, aliquot the solution into convenient volumes and place immediately at -20 °C.
2. Thaw an aliquot of MESG substrate before use by placing it in a 37 °C water bath until just melted (not more than 5 min). Vortex vigorously and immediately chill the solution by placing it on ice.
   Note: The solution is stable for at least 4 h on ice at pH 7.5. If left at room temperature, the half-life of MESG is about 4 h at pH 8.0 and 40 h at pH 6.0. Do not refreeze leftover MESG substrate.
3. Prepare enzyme purine nucleoside phosphorylase (Component B) stock by adding 500 µl of dH_2O to a vial to prepare a 100 U/ml stock solution and store at 4 °C.
4. Prepare recombinant ATPase protein (test protein) stock.

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Note: Use different concentrations of test protein to analyze ATPase activity.

5. Prepare 1 mM ATP stock. Make 10 mM ATP stock by adding 0.05 g of ATP (disodium salt) in 10 ml of 25 mM Tris-Cl (pH 8.0) buffer (250 µl of 1 M Tris-Cl pH 8.0 in 9.75 ml sterilized water). Dilute to 1 mM ATP stock by adding 1 ml of 10 mM ATP in 9 ml of 25 mM Tris-Cl (pH 8.0).
6. Dilute a portion of the 50 mM potassium phosphate monobasic (KH_2PO_4), a phosphate standard, 100-fold with dH_2O to generate a 500 µM solution. Further, dilute a portion of 500 µM solution to make 1 µM KH_2PO_4, working solution.

Phosphate Standard curve

1. Prepare 10, 20, 30, 40, and 50 nmol of inorganic phosphate standard with 1 µM KH_2PO_4 in corresponding well to the final volume of 300 µl reaction mix as per Table 2 below:

Table 2. Phosphate standard curve reaction mix composition

Well	Inorganic phosphate nmol	(KH_2PO_4 1 µM) µl	20x Reaction Buffer µl	MESG substrate µl	Water µl	purine nucleoside phosphorylase µl
Blank	0	0	15	60	222	3
SD1	10	3	15	60	219	3
SD2	20	6	15	60	216	3
SD3	30	9	15	60	213	3
SD4	40	12	15	60	210	3
SD5	50	15	15	60	207	3

2. Use a 96-well microtiter plate to perform the assay.
3. Start the reaction by the addition of final component purine nucleoside phosphorylase and incubate for 30 min at 22 °C.
4. Read the absorbance at 360 nm. Correct the absorbance by subtracting blank from each standard.
5. Plot Phosphate standard curve in Excel by plotting known inorganic phosphate concertation on X-axis and ABS_{360} on Y-axis and obtain trendline and equation. The representative data is shown in Figure 3.

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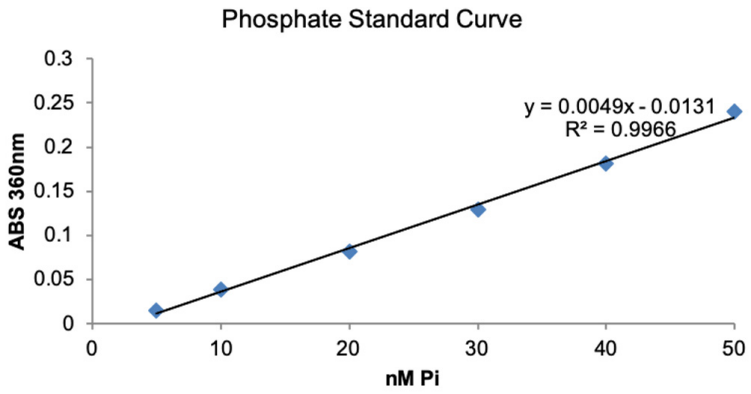

Figure 3. Phosphate standard curve

ATPase reaction

1. Use a 96-well microtiter plate to perform the assay. If the plate reader is not available, use 1 ml of assay mix to perform the assay in cuvettes.
2. Make triplicate for all reactions.
3. Prepare assay mix by mixing the stock solution to make 300 µl assay mix for plate reader in 96-well plates and 1 ml assay mix to use in the cuvette as described in Table 3 below:

Table 3. ATPase assay reaction mix composition

Stock solution	Assay mix (300 µl)	If 1 ml of assay mix
20x Reaction Buffer	15 µl	50 µl
MESG substrate solution	60 µl	200 µl
Recombinant test protein/enzyme (0.5-2 µg)	100 µl	500 µl
Purine nucleoside phosphorylase (PNP) (1 U)	3 µl	10 µl
Water	Make up the volume to 300 µl	Make up the volume to 1 ml

4. Prepare blank in a microtiter plate well by adding water instead of recombinant ATPase test protein.
5. Prepare the sample volume of recombinant ATPase test protein ranging from 0.5 to 2 µg of protein in water to the total volume to 100 µl.
6. Prepare test samples well by adding 100 µl of recombinant ATPase test protein prepared above.
6. Prepare positive control microtiter plate well by adding 0.5 units of ATPase (Sigma) instead of recombinant ATPase test protein.
7. Incubate the reaction at 22 °C for 10 min in the plate reader.
8. Start the reaction by adding 25 µl of ATP (1 mM) as a substrate in each microtiter plate well and mix in the plate reader. If using 1 ml cuvette the amount of ATP needs to be increased accordingly (83 µl).

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9. Read absorbance in a microtiter plate reader at 360 nm at 0 min and then 10 min intervals for one hour or until saturation point has reached at 22 °C. Incubate the plate in the microtiter plate reader.

Data analysis

1. Calculate the $\Delta_{A360nm}/min_{test}$ (ABS_{360} at saturation point (min) - ABS_{360} at 0 min for test samples).
2. Calculate the $\Delta_{A360nm}/min_{blank}$ (ABS_{360} at saturation point (Min) - ABS_{360} at 0 min for blank).
3. Calculate the $\Delta\Delta_{ABS360} = (\Delta_{ABS360nm}/min_{test} - \Delta_{ABS360nm}/min_{blank})$. Calculate Average and standard error using all three replicates in each reaction.
4. Plot $\Delta\Delta_{ABS360}$ on Y-axis vs recombinant protein concertation on X-axis. The representative data for $\Delta\Delta_{ABS360}$ vs recombinant test protein are shown in Figure 4.

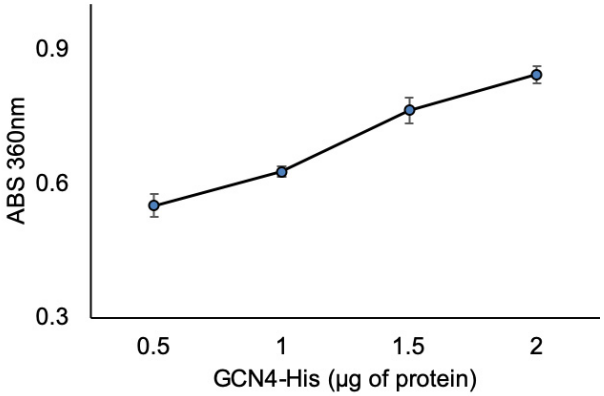

Figure 4. ABS_{360nm} vs. test (GCN4-His) protein concentration

5. Calculate the amount of inorganic phosphate (Pi) using the phosphate standard curve trendline equation (Figure 3) by substituting y with $\Delta\Delta_{ABS360}$.
6. Calculate ATPase activity using the following formula:

   ATPase Activity =Pi/(t × V) × D = nmol/min/µl = mU/µl = U/ml

   where,
   Pi is the inorganic phosphate amount (nmol) from the standard curve (obtained in step 5),
   t is the reaction time (min) (time to reach the saturation point),
   V is the sample volume (100 µl of recombinant ATPase test protein) added into the reaction well (µl),
   D is the sample dilution factor (100 µl/300 µl) = 0.34.
   Unit Definition: One unit of ATPase is the amount of enzyme that will generate 1.0 µmol of phosphate per min.
7. Plot ATPase activity (U/ml) on Y-axis vs recombinant test protein concentration on X-axis.

8. To calculate specific activity, divide the U/ml by the amount of protein present in the sample (converted to mg/ml).

Recipes

1. 1 M Tris-Cl (pH-8.0)
   Tris base 12.11 g
   Deionized H_2O 80 ml
   Adjust pH to 8.0 with HCl
   Make up volume to 100 ml with deionized water
   Autoclave at 121 °C for 15 min

Acknowledgments

This work was supported by funds from the Noble Research Institute, LLC. This protocol was derived from Kaundal *et al.*, 2017 manuscript.

Competing interests

The authors declare no financial or non-financial competing interests.

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