Revised: 24–November–2003

EnzChek® Pyrophosphate Assay Kit (E-6645)

Quick Facts

Storage upon receipt:

- ≤–20°C

Notes: Rinse all cuvettes thoroughly with deionized water between spectrophotometric measurements to prevent P_i carryover.

Introduction

Molecular Probes' EnzChek® Pyrophosphate Detection Kit provides a fast, convenient and inexpensive spectrophotometric method for measuring the inorganic pyrophosphate (PP_i) produced by a number of biochemical reactions, such as DNA and RNA polymerizations, cyclic AMP formation by the enzyme adenylate cyclase and the enzymatic activation of fatty acids to form their coenzyme A esters.[1] The EnzChek Pyrophosphate Assay Kit is a modification of our EnzChek Phosphate Assay Kit (E-6646), which is based on the method originally described by Webb.[2] In the presence of inorganic phosphate (P_i), the substrate 2-amino-6-mercapto-7-methylpurine ribonucleoside (MESG) is converted enzymatically by purine nucleoside phosphorylase (PNP) to ribose 1-phosphate and 2-amino-6-mercapto-7-methylpurine (Figure 1). Enzymatic conversion of MESG results in a shift in absorbance maximum from 330 nm for the substrate to 360 nm for the product.

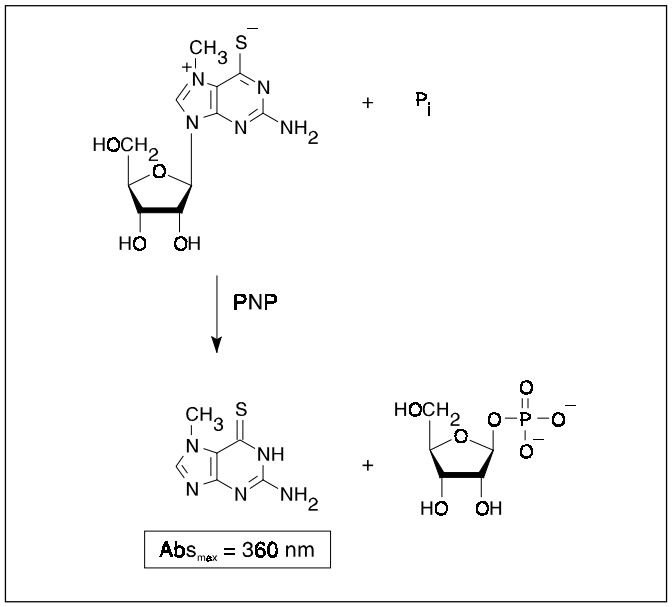

Figure 1. *Enzymatic conversion of 2-amino-6-mercapto-7-methylpurine ribonucleoside (MESG) to ribose 1-phosphate and 2-amino-6-mercapto-7-methylpurine by purine ribonucleoside phosphorylase (PNP). The accompanying change in the absorption maximum at 360 nm allows quantitation of inorganic phosphate (P_i) consumed in the reaction.*

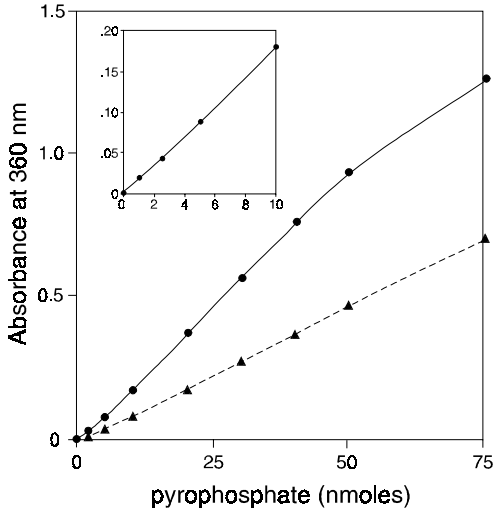

Figure 2. *Comparison of standard curves using $Na_2P_2O_7$ as the source of pyrophosphate (upper curve, closed circles) and KH_2PO_4 as the source of phosphate (lower curve, closed triangles). Reaction volumes were 1 mL and the absorbances at 360 nm have been corrected for background absorbance. The inset shows an enlargement of the pyrophosphate curve, demonstrating the sensitivity of the assay; the units are the same.*

The EnzChek Pyrophosphate Assay Kit includes the enzyme inorganic pyrophosphatase, which catalyzes conversion of PP_i present into two equivalents of P_i. The P_i is then consumed by the MESG/PNP reaction and detected by an increase in absorbance at 360 nm. Additional sensitivity is gained by the amplification of one molecule of PP_i to two molecules of P_i. The assay can detect as little as 1 nanomole of PP_i in 1 mL reactions (Figure 2) and can be performed over a pH range of 6.5 to 8.5.[2] The MESG/PNP reaction has been used for continuous spectrophotometric determination of aminoacyl-tRNA synthetase activity.[3] In addition, we have tested the ability of this assay to detect the PP_i liberated by the activities of acetyl-CoA synthetase (Figure 3) and luciferase (Figure 4). In these experiments, we have detected as few as 5×10^{-5} U of acetyl-CoA synthetase activity and 10^{-2} U of luciferase activity.

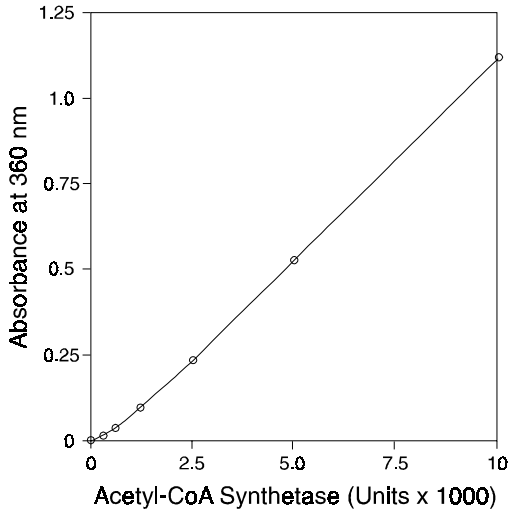

Figure 3. *Measurement of acetyl-CoA synthetase activity. The following reagents were combined in 1 mL reaction volumes: 0.4 mM ATP, 0.4 mM CoA, 0.4 mM sodium acetate, 0.2 mM MESG, 1 U purine nucleoside phosphorylase, 0.01 U pyrophosphatase, 1X reaction buffer and various dilutions of acetyl-CoA synthetase. After incubating at 22°C for 30 minutes, the absorbance at 360 nm was measured and corrected for absorbance at 360 nm of a control reaction lacking acetyl-CoA synthetase.*

Materials

Kit Contents

- **MESG substrate** (Component A), 6.3 mg (20 µmoles)
- **Purine nucleoside phosphorylase (PNP)** (Component B), two vials, each containing 50 U of lyophilized enzyme; one Unit of PNP will cause the phosphorolysis of 1.0 µmole of inosine to hypoxanthine and ribose 1-phosphate per minute at pH 7.4 at 25°C
- **20X reaction buffer** (Component C), 10 mL of 1.0 M Tris-HCl, 20 mM $MgCl_2$, pH 7.5, containing 2 mM sodium azide
- **Pyrophosphate standard** (Component D), 500 µL of 50 mM $Na_4P_2O_7$, containing 2 mM sodium azide
- **Inorganic pyrophosphatase** (Component E), one vial containing 6 U of lyophilized enzyme; one Unit of inorganic phosphatase will liberate 1.0 µmole of inorganic orthophosphate per minute at pH 7.2 at 25°C

This kit provides sufficient reagents for approximately 100 assays, each performed in a 1 mL volume.

Storage

Upon receipt, this kit should be stored at ≤-20°C. Allow reagents to warm to room temperature before opening the vials. When stored properly, these reagents are stable for six months to one year. Reconstituted purine nucleoside phosphorylase may be stored at 4°C for at least one month. Diluted inorganic pyrophosphatase (see step 1.6) may be stored at 4°C for at least one week. Reconstituted MESG may be stored at ≤-20°C for at least one month.

Important note: Due to the high sensitivity of this assay for P_i, it is extremely important to use P_i-free laboratory ware and reagents. Rinse all cuvettes thoroughly with deionized water (dH_2O) between spectrophotometric measurements to prevent P_i carryover.

Experimental Protocol

Reagent Preparation

1.1 Prepare a 1 mM stock solution of MESG by adding 20 mL of dH_2O directly to the bottle containing the MESG substrate (Component A). Immediately after dissolving, aliquot the MESG solution in convenient volumes (each standard reaction requires 200 µL, see *Standard Reaction*) and place immediately at -20°C.

1.2 Thaw an aliquot of MESG substrate immediately before use by placing in a 37°C water bath until just melted (no more than 5 minutes).

1.3 Vortex vigorously, then place on ice. This solution is stable for at least 4 hours on ice at pH 7.5. If left at room temperature, the half-life of MESG is about 4 hours at pH 8.0 and 40 hours at pH 6.0.[1] We do not recommend refreezing leftover MESG substrate.

1.4 Prepare 1X reaction buffer from the 20X reaction buffer provided (Component C).

1.5 Add 0.2 mL of dH_20 to the vial of inorganic pyrophosphatase (Component E) to prepare a 30 U/mL stock solution. Store at 4°C. Prior to use, dilute an aliquot of the inorganic pyrophosphatase stock solution tenfold by mixing with 1X reaction buffer to generate a 3 U/mL working stock solution; *do not dilute inorganic phosphatase directly into 20X reaction buffer.*

1.6 Add 0.5 mL of dH_2O to a vial of purine nucleoside phosphorylase (Component B) to prepare a 100 U/mL stock solution. Store at 4°C.

1.7 Dilute a portion of the pyrophosphate standard (Component D) 100-fold with dH_2O to generate a 500 µM working

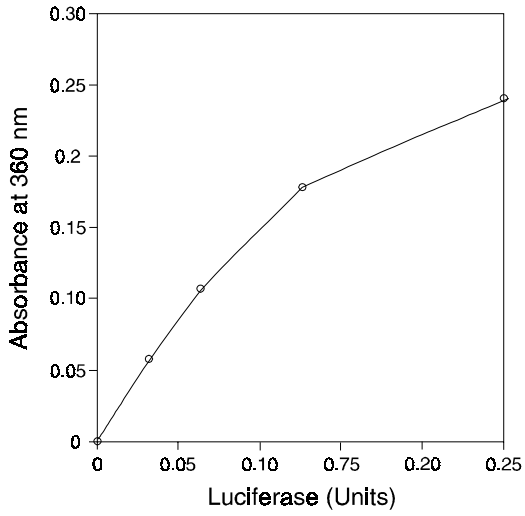

Figure 4. *Measurement of luciferase activity. The following reagents were combined in 1 mL reaction volumes: 0.1 mM luciferin, 1 mM ATP, 1 mM DTT, 5 mM magnesium sulfate, 0.1 mM EDTA, 0.2 mM MESG, 1 U purine nucleoside phosphorylase, 0.01 U pyrophosphatase, 25 mM tricine, pH 7.8, and various dilutions of luciferase. After incubating at 22°C for 30 minutes, the absorbance at 360 nm was measured and corrected for background absorbance at 360 nm.*

solution. A 1 mL volume of this working solution is usually sufficient to generate a standard curve.

Standard Reaction

The EnzChek Pyrophosphate Assay Kit can be used for the quantitation of PP_i in solution (Figure 4) or for the continuous determination of PP_i released in enzymatic reactions. In either case, the standard 1 mL reaction mixture contains:

- 730 µL - x µL dH_2O (to create a final volume of 1.0 mL)
- 50 µL 20X reaction buffer
- 200 µL MESG substrate solution (from step 1.3)
- x µL sample to be analyzed for pyrophosphate content
- 10 µL purine nucleoside phosphorylase (1 U, from step 1.4)
- 10 µL inorganic pyrophosphatase (0.03 U, from step 1.5)

Generally, for the routine determination of pyrophosphate in solution, the components are added to the reaction vial or cuvette in the order listed above; the reactions are started by the addition of the final component (inorganic pyrophosphatase) and incubated for 30–60 minutes at 22°C. For the continuous determination of PP_i released in reactions, special conditions apply; see *Inorganic Pyrophosphate Released Continuously by Enzymatic Reaction*.

Standard Curve for Inorganic Pyrophosphate

A standard curve for the pyrophosphate assay can be generated using the pyrophosphate standard as a source of PP_i. The linear range of the assay for PP_i extends from 1 µM to about 75 µM (see Figure 2).

2.1 Add variable amounts of the pyrophosphate standard working solution (from step 1.6) to the standard reaction mixture (see *Standard Reaction*). For example, each 10 µL of 500 µM pyrophosphate standard solution added to the 1 mL reaction contributes 5 µM PP_i to the reaction mixture. Always include a no-pyrophosphate control: standard reaction mix without pyrophosphate standard added, measured against a 1X reaction buffer blank at 360 nm.

2.2 Mix well and incubate the reaction mixtures for 30–60 minutes at 22°C.

2.3 Read the absorbance at 360 nm. Subtract the background absorbance determined for the no-pyrophosphate control from each sample before plotting the curve.

Inorganic Pyrophosphate in Solutions

The concentration of PP_i in solutions can be determined accurately by using the standard reaction mixture in conjunction with a standard curve for PP_i (see *Curve for Inorganic Pyrophosphate*). Likewise, the PP_i generated in discontinuous reactions, i.e. reactions that are terminated or run to completion before the PP_i assay, can be measured in the standard reaction mixture. It is important, however, to consider the possibility that experimental reagents may be contaminated with P_i (or less likely with PP_i) because P_i is the species required for the enzymatic conversion of MESG and subsequent absorbance increase. The individual reagents can be pretested for their P_i content in a reaction similar to the standard reaction, but with the inorganic pyrophosphatase omitted. Contaminating P_i (or PP_i) often can be tolerated, and subtracted out, by using the best possible controls. It is also important to generate the standard curve using the same buffer conditions as your samples. The assay can be performed over a pH range of 6.5 to 8.5.

Inorganic Pyrophosphate Released Continuously by Enzymatic Reaction

The EnzChek Pyrophosphate Assay Kit can be used to follow the kinetics of PP_i released continuously in an enzymatic reaction. In these measurements it is particularly important to monitor reagents and enzymes for the possibility of P_i contamination (see *Inorganic Pyrophosphate in Solutions*). For example, if the enzyme you are studying contains contaminating P_i, you will have to decide if meaningful data can be obtained using this assay. Contaminating P_i can often be reduced to submicromolar levels by using the MESG/PNP reaction as a "P_i mop."[1] In this procedure, the contaminated reagent (e.g., the enzyme) is preincubated in the assay mixture before the kinetic reaction is started (e.g., by adding the substrate). The following protocol is for a hypothetical "experimental enzyme," contaminated with trace P_i, and its "experimental substrate;" your experimental situation may differ. In addition, for continuous assay of PP_i, the activity of a PP_i-generating enzyme should not exceed the capacity of the inorganic pyrophosphatase in the reaction; the appropriate amount of experimental enzyme added will have to be determined empirically.

3.1 Set up each reaction as follows, omitting the experimental substrate:

- 730 µL - x µL - y µL dH_2O (to create final volume of 1.0 mL)
- 50 µL 20X reaction buffer
- 200 µL MESG substrate solution (from step 1.3)
- 10 µL purine nucleoside phosphorylase (1 U, from step 1.4)
- 10 µL inorganic pyrophosphatase (0.03 U, from step 1.5)
- x µL experimental enzyme

where y µL is the volume of the experimental substrate, to be added later.

3.2 Preincubate for 10 minutes at 22°C.

3.3 Add y µL of experimental substrate and mix well; in a parallel control assay, add y µL of buffer instead of substrate.

3.4 Immediately begin reading absorbance at 360 nm as a function of time for both the experimental reaction and the control reaction.

3.5 For data analysis, subtract the values determined for the no-substrate control from the corresponding values for the experimental reaction.

References

1. Anal Biochem 243, 41 (1996); **2.** Proc Natl Acad Sci USA 89, 4884 (1992); **3.** Nucleic Acids Res 23, 2886 (1995).

Product List *Current prices may be obtained from our Web site or from our Customer Service Department.*

Cat #	Product Name	Unit Size
E-6645	EnzChek® Pyrophosphate Assay Kit *100 assays*	1 kit

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Further information on Molecular Probes' products, including product bibliographies, is available from your local distributor or directly from Molecular Probes. Customers in Europe, Africa and the Middle East should contact our office in Leiden, the Netherlands. All others should contact our Technical Assistance Department in Eugene, Oregon.

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