Revised: 21–June–2002

EnzChek® Phosphate Assay Kit (E-6646)

Quick Facts

Storage upon receipt:

- –20°C
- Desiccate

Abs$_{max}$ of substrate: 330 nm
Abs$_{max}$ of product: 360 nm

Introduction

Molecular Probes' EnzChek® Phosphate Assay Kit provides a fast and sensitive spectrophotometric method for the quantitation of inorganic phosphate (P_i) in solution, including P_i released from enzymatic reactions. This kit enables continuous assay of reactions that generate P_i such as those catalyzed by ATPases and GTPases. The EnzChek phosphate assay is based on a method originally described by Webb.[1] In the presence of P_i, the substrate 2-amino-6-mercapto-7-methylpurine riboside (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 spectrophotometric shift in maximum absorbance from 330 nm for the substrate to 360 nm for the product. Sensitivity of the assay is in the range of 2 to 150 µM P_i (2 to 150 nanomoles P_i in a 1 mL volume), and the reaction can be performed over a pH range of 6.5 to 8.5, with the proper controls.[1]

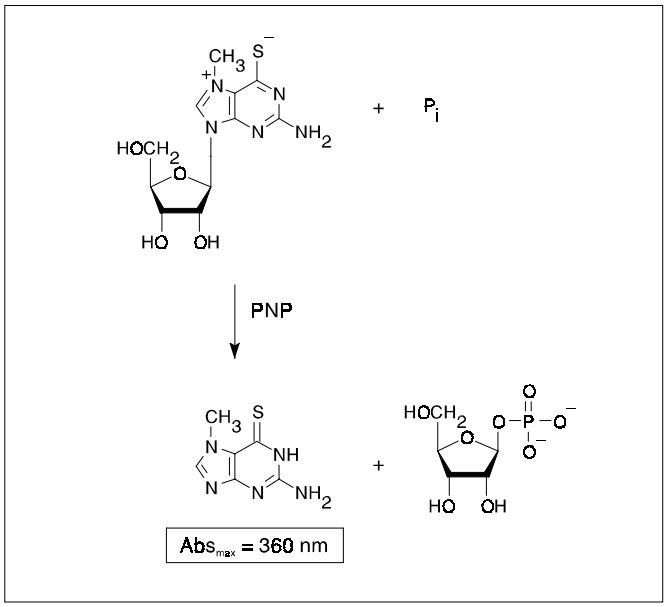

Figure 1. *Enzymatic conversion of 2-amino-6-mercapto-7-methylpurine riboside (MESG) to ribose 1-phosphate and 2-amino-6-mercapto-7-methylpurine by purine nucleoside phosphorylase (PNP). The accompanying change in absorption at 360 nm allows quantitation of inorganic phosphate (P_i) consumed in the reaction.*

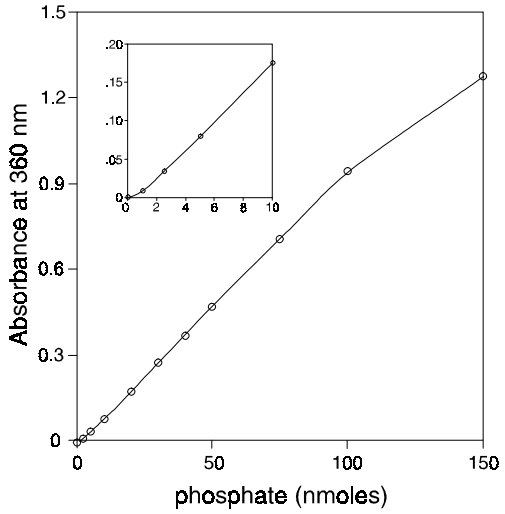

Figure 2. *Standard curve where KH_2PO_4 was used as the source for inorganic phosphate. Reaction volumes were 1 mL, and the absorbances at 360 nm were corrected for background absorbance. The inset shows an enlargement of the standard curve, demonstrating the lower range of the assay; the units are the same.*

The EnzChek phosphate reaction is sufficiently fast and quantitative that MESG/PNP can be used for stopped-flow kinetic experiments.[1] We have used this assay to measure P_i in solution (Figure 2) and to monitor P_i produced by ATPase (Figure 3), glycerokinase and 5′-nucleotidase. The assay has also been used to detect P_i contamination in the presence of high concentrations of acid-labile phosphates[2] and to monitor the kinetics of phosphate release of a wide variety of enzymes including:

- actin-activated myosin ATPase[1]
- aspartate transcarbamylase[3]
- dethiobiotin synthetase[4]
- glycerol kinase[1]
- glycogen phosphorylase[5]
- GTPases[6,7]
- *myo*-inositol monophosphatase[8]
- phospholysine and phosphohistidine phosphatases[9,10]

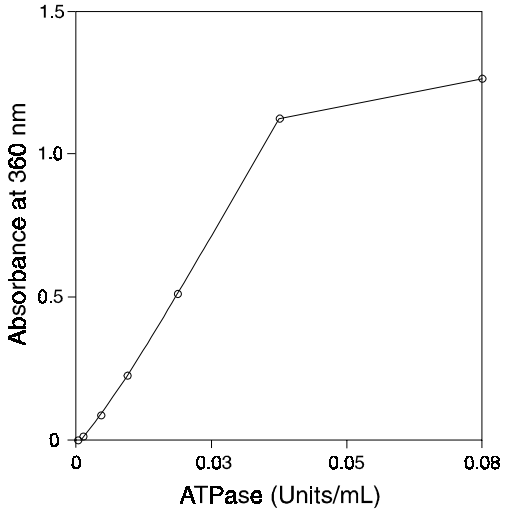

Figure 3. *Measurement of ATPase activity. The following reagents were combined in 1 mL reaction volumes: 400 µM ATP, 1 mM KCl, 1 mM NaCl, 1 U of purine nucleoside phosphorylase, various dilutions of ATPase and 1X reaction buffer. After incubation at 22°C for 30 minutes, the absorbance at 360 nm was measured and corrected for background absorbance.*

- phosphorylase *a* phosphatase [11]
- phosphorylase kinase [12]
- protein serine phosphatase [13]

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
- **Phosphate standard** (Component D), 500 µL of 50 mM KH_2PO_4, containing 2 mM sodium azide

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

Storage and Handling

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. 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). Extensive mixing (e.g. 10 minutes) is required to fully dissolve the MESG. Do not heat. 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 (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 Add 500 µL of dH_2O to a vial of purine nucleoside phosphorylase (Component B) to prepare a 100 U/mL stock solution. Store at 4°C.

1.5 Dilute a portion of the phosphate standard 100-fold with dH_2O to generate a 500 µM working solution. A 1 mL volume of this working solution is usually sufficient to generate a standard curve.

Standard Reaction

The EnzChek Phosphate Assay Kit can be used for the quantitation of P_i in solution or for the continuous determination of P_i released in enzymatic reactions. In either case, the standard 1 mL reaction mixture contains:

- 740 µL – x µL dH_2O (to create a final reaction 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 phosphate content
- 10 µL purine nucleoside phosphorylase (1 U, from step 1.4)

Generally, for the routine determination of phosphate 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 (the purine nucleoside phosphorylase) and incubated for 30 minutes at 22°C. For the continuous determination of P_i released in reactions, special conditions apply; see *Inorganic Phosphate Released Continuously by Enzymatic Reaction.*

Standard Curve for Inorganic Phosphate

A standard curve for the phosphate assay may be generated using the phosphate standard as a source of P_i. The linear range of the assay for P_i extends from 2 µM to about 150 µM (see Figure 2).

2.1 Add variable amounts of the phosphate standard working solution (from step 1.5) to the standard reaction mixture (see

Standard Reaction). For example, each 10 µL of 500 µM phosphate standard solution added to the 1 mL reaction contributes 5 µM P_i to the reaction mixture. Always include a no-phosphate control: standard reaction mix (see *Standard Reaction*) without phosphate standard added, measured against a 1X reaction buffer blank at 360 nm.

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

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

Inorganic Phosphate in Solutions

The concentration of P_i in solutions can be determined accurately by using the standard reaction mixture (see *Standard Reaction*) in conjunction with a standard curve for P_i (see *Standard Curve for Inorganic Phosphate*). Likewise, the P_i generated in discontinuous reactions, i.e. reactions that are terminated or run to completion before the P_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. The individual reagents can be pretested in the assay for their P_i content. Contaminating P_i can often 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 Phosphate Released Continuously by Enzymatic Reaction

The EnzCheck Phosphate Assay Kit can be used to follow the kinetics of P_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 Phosphate 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 situation may differ.

3.1 Set up the reaction mixtures omitting the experimental substrate:

- 740 µL – x µL – y 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)
- 10 µL purine nucleoside phosphorylase (1 U, from step 1.4)
- 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. Proc Natl Acad Sci USA 89, 4884 (1992); **2.** Anal Biochem 230, 173 (1995); **3.** Anal Biochem 218, 449 (1994); **4.** Biochemistry 34, 10976 (1995); **5.** Anal Biochem 221, 348 (1994); **6.** Biochemistry 34, 15592 (1995); **7.** Biochem J 287, 555 (1992); **8.** Biochem J 307, 585 (1995); **9.** Anal Biochem 222, 14 (1994); **10.** Biochem J 296, 293 (1993); **11.** Anal Biochem 226, 68 (1995); **12.** Anal Biochem 230, 55 (1995); **13.** Biochemistry 33, 2380 (1994).

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

Cat #	Product Name	Unit Size
E-6646	EnzChek® Phosphate Assay Kit *100 assays*	1 kit
E-6645	EnzChek® Pyrophosphate Assay Kit *100 assays*	1 kit

Contact Information

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.

Please visit our Web site — **www.probes.com** — for the most up-to-date information

Molecular Probes, Inc.
29851 Willow Creek Road, Eugene, OR 97402
Phone: (541) 465-8300 ● Fax: (541) 344-6504

Customer Service: 7:00 am to 5:00 pm (Pacific Time)
Phone: (541) 465-8338 ● Fax: (541) 344-6504 ● order@probes.com

Toll-Free Ordering for USA and Canada:
Order Phone: (800) 438-2209 ● Order Fax: (800) 438-0228

Technical Assistance: 8:00 am to 4:00 pm (Pacific Time)
Phone: (541) 465-8353 ● Fax: (541) 465-4593 ● tech@probes.com

Molecular Probes Europe BV
PoortGebouw, Rijnsburgerweg 10
2333 AA Leiden, The Netherlands
Phone: +31-71-5233378 ● Fax: +31-71-5233419

Customer Service: 9:00 to 16:30 (Central European Time)
Phone: +31-71-5236850 ● Fax: +31-71-5233419
eurorder@probes.nl

Technical Assistance: 9:00 to 16:30 (Central European Time)
Phone: +31-71-5233431 ● Fax: +31-71-5241883
eurotech@probes.nl

Molecular Probes' products are high-quality reagents and materials intended for research purposes only. These products must be used by, or directly under the supervision of, a technically qualified individual experienced in handling potentially hazardous chemicals. Please read the Material Safety Data Sheet provided for each product; other regulatory considerations may apply.

Several of Molecular Probes' products and product applications are covered by U.S. and foreign patents and patents pending. Our products are not available for resale or other commercial uses without a specific agreement from Molecular Probes, Inc. We welcome inquiries about licensing the use of our dyes, trademarks or technologies. Please submit inquiries by e-mail to busdev@probes.com. All names containing the designation ® are registered with the U.S. Patent and Trademark Office.