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EnzChek® Phosphatase Assay Kit (E12020)

Quick Facts

Storage upon receipt:
- ≤–20°C
- Protect from light

Ex/Em of reaction product: ~358/455 nm

Introduction

The EnzChek® Phosphatase Assay Kit can be used to continuously detect phosphatase activity at neutral, alkaline, or acidic pH. The kit contains Molecular Probes' patented 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP) substrate (D6567, D22065). Because the reaction product of DiFMUP does not require addition of base to the reaction medium prior to measuring the fluorescence, DiFMUP can be used for the continuous assay of phosphatases with neutral, alkaline, or moderately acidic pH optima.

DiFMUP is a fluorinated relative of 4-methylumbelliferyl phosphate (MUP), a fluorogenic substrate that has most often been used to measure alkaline phosphatase activity.[1,2] One problem with MUP is the requirement for an alkaline pH to maximize the fluorescence signal, which precludes its use in continuous assays at neutral or moderately acidic pH. This problem results from the high pK_a of the reaction product, 4-methylumbelliferone ($pK_a \approx 8$). The DiFMUP substrate overcomes this problem, since its reaction product, 6,8-difluoro-4-methylumbelliferone, has a pK_a of ~4.7. Thus, DiFMUP is about 100 times more sensitive than MUP for the detection of prostatic acid phosphatase at pH 5.5 (Figure 1). The EnzChek Phosphatase Assay Kit is perfect for the continuous assay of prostatic acid phosphatase (Figure 2), protein phosphatase 1 or almost any other phosphatase that can be assayed with nonprotein-based substrates such as MUP or 4-nitrophenyl phosphate (PNPP). Each kit contains sufficient substrate for ~1000 assays in a fluorescence mi-croplate reader, using a reaction volume of 100 µL per assay. The kit also contains reaction buffer, 6,8-difluoro-7-hydroxy-4-methylcoumarin for use as a reference standard and potato acid phosphatase for use as a positive control (Figure 3). Fluorescence can be measured in a fluorescence microplate reader or a standard fluorometer — the reaction product has excitation/emission maxima of ~358/455 nm.

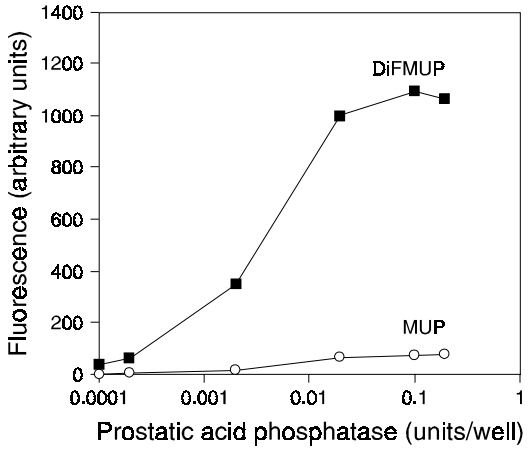

Figure 1. *Comparison of DiFMUP with MUP for the detection of acid phosphatase activity at pH 5.5. Increasing amounts of prostatic acid phosphatase from human semen were reacted with 100 µM DiFMUP, the substrate in the EnzChek Phosphatase Assay Kit, or 100 µM MUP, in 100 mM sodium acetate, pH 5.5, at room temperature. Fluorescence was measured after 60 minutes in a fluorescence microplate reader using excitation at 360 ± 20 nm and emission detection at 460 ± 20 nm.*

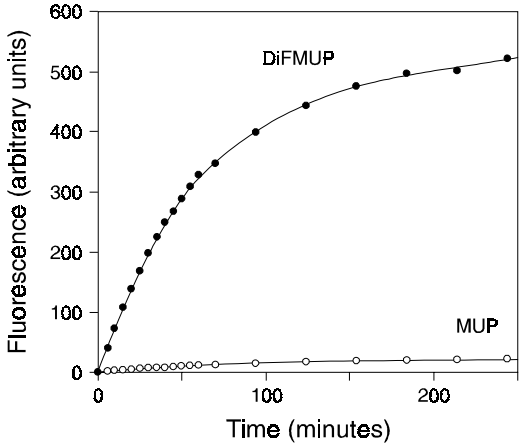

Figure 2. *Time course of the reaction of prostatic acid phosphatase with DiFMUP and MUP. Prostatic acid phosphatase from human semen (0.002 units) was reacted with 100 µM DiFMUP or 100 µM MUP in 100 mM sodium acetate, pH 5.5, at room temperature. Fluorescence was measured at the indicated times, as described for Figure 1.*

Materials

Kit Components

- **DiFMUP substrate** (MW = 292.1, Component A), five vials, each containing 584 µg
- ***N,N*-dimethylformamide (DMF)** (Component B), 1.5 mL
- **5X Reaction Buffer** (Component C), 28 mL of 0.5 M sodium acetate, pH 5.0

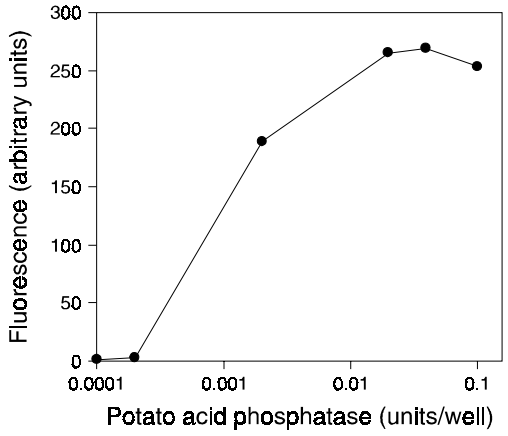

Figure 3*. Detection of potato acid phosphatase using the EnzChek Phosphatase Assay Kit. Increasing amounts of potato acid phosphatase were reacted with 100 µM DiFMUP, as described in the kit protocol. Fluorescence was measured after 60 minutes in a fluorescence microplate reader using excitation at 360 ± 20 nm and emission detection at 460 ± 20 nm.*

- **Acid phosphatase from potato** (Component D), 5 U, where one unit is defined as the amount of enzyme that will hydrolyze 1.0 µmole of *p*-nitrophenyl phosphate per minute at pH 4.8 at 37°C
- **6,8-Difluoro-7-hydroxy-4-methylcoumarin reference standard** (MW = 212.2, Component E), 212 µg

Each kit contains sufficient reagents for performing approximately 1000 assays, using a reaction volume of 100 µL per assay.

Storage

Upon receipt, the EnzChek Phosphatase Assay Kit should be stored at ≤–20°C. The DiFMUP substrate should be protected from light.

Stock Solution Preparation

1.1 Prepare a 10 mM stock solution of DiFMUP: Bring one vial of DiFMUP substrate (Component A) and the vial of DMF (Component B) to room temperature. Add 200 µL of DMF directly to the vial of DiFMUP. This 200 µL volume is sufficient for performing ~200 assays using the protocol described below. If stored desiccated at ≤–20°C, protected from light, the DiFMUP stock solution should remain stable for approximately one month.

1.2 Prepare a 1X working solution of Reaction Buffer by adding 5 mL of 5X Reaction Buffer stock solution (Component C) to 20 mL of deionized water (dH_2O). This 25 mL volume of 1X Reaction Buffer is sufficient for approximately 200 assays of 100 µL each, with a 5 mL excess for making stock solutions and dilutions.

1.3 Prepare a 10 U/mL stock solution of potato acid phosphatase by adding 0.5 mL of 1X Reaction Buffer directly to the vial of potato acid phosphatase (Component D). After use, the remaining solution should be divided into small aliquots and stored frozen at ≤–20°C.

1.4 If desired, prepare a 10 mM stock solution of 6,8-difluoro-7-hydroxy-4-methylcoumarin reference standard by adding 100 µL of DMF directly to the vial of 6,8-difluoro-7-hydroxy-4-methylcoumarin solid (Component E). This solution can be used to prepare a standard curve to determine the moles of product produced in the DiFMUP-containing reactions. This stock solution should be stored frozen at ≤–20°C, protected from light.

Experimental Protocol

The following procedure is designed for use with a fluorescence microplate reader. For use with a standard fluorometer, volumes should be increased accordingly. The Reaction Buffer is included for your convenience and is optimized for the potato acid phosphatase control enzyme. Other buffers can be substituted, if desired. Please note that the use of phosphate-containing buffers is not recommended.

2.1 Just prior to performing the assay, prepare a 200 µM DiFMUP working solution. For example, to prepare sufficient working solution for 200 assays, add the entire amount (200 µL) of 10 mM DiFMUP stock solution (prepared in step 1.1) to 9.8 mL of 1X Reaction Buffer or the buffer of your choice.

2.2 Pipet 50 µL of the 200 µM DiFMUP working solution into each microplate well.

2.3 Dilute the phosphatase-containing samples in 1X Reaction Buffer or the buffer of your choice. Please note that the final concentration will be twofold lower.

2.4 If a positive control is desired, prepare a 1 U/mL working solution of potato acid phosphatase by diluting the 10 U/mL potato acid phosphatase stock solution (prepared in step 1.3) tenfold in 1X Reaction Buffer.

2.5 Add 50 µL of each sample or control to the substrate-containing microplate wells. Use 50 µL of 1X Reaction Buffer as a negative control.

2.6 Incubate the samples at room temperature, protected from light, for the desired length of time. Potato acid phosphatase activity can be detected in as little as 15 minutes. Because the assay is continuous, measurements can be made on the same samples at multiple time points.

2.7 If desired, prepare a 6,8-difluoro-7-hydroxy-4-methylcoumarin standard curve: Dilute the appropriate amount of 10 mM 6,8-difluoro-7-hydroxy-4-methylcoumarin stock solution (prepared in step 1.4) into 1X Reaction Buffer or the buffer of your choice to yield 6,8-difluoro-7-hydroxy-4-methylcoumarin solutions ranging in concentration from 0–100 µM. Pipet 100 µL of each standard into additional microplate wells at any time prior to measuring the fluorescence.

2.8 Measure the fluorescence using excitation at ~360 nm and emission detection at ~460 nm. The reaction product has excitation and emission maxima of approximately 358 nm and 455 nm, respectively.

References

1. Biologicals 18, 331 (1990); **2.** Biochem J 97, 95 (1965).

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

Cat #	Product Name	Unit Size
D6567	6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP)	5 mg
D22065	6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP) *packaged for high-throughput screening*	10 x 10 mg
E12020	EnzChek® Phosphatase Assay Kit *1000 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.

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