

EnzChek® Ultra Phytase Assay Kit

Catalog no. E33701

Table 1. Contents and storage information.

Material	Amount	Storage	Stability
Amplex® UltraRed reagent, MW = ~300 (Component A)	5 vials, each containing 0.18 mg	<ul style="list-style-type: none">• ≤-20°C• Desiccate• Protect from light	When stored as directed this kit is stable for 1 year.
Dimethylsulfoxide (DMSO), anhydrous (Component B)	500 µL		
10X Reaction Buffer (Component C)	28 mL of 1 M sodium acetate, pH 5.5		
Phytic acid, dodecasodium salt hydrate, FW = 923.8 (Component D)	65 mg, 85% by weight		
Maltose phosphorylase, recombinant from <i>Escherichia coli</i> (Component E)	150 U*		
Glucose oxidase, from <i>Aspergillus niger</i> (Component F)	200 U†		
Horseradish peroxidase (HRP) (Component G)	30 U‡		
Maltose, monohydrate, MW = 360.3 (Component H),	20 mg		
Phosphate standard (Component I)	500 µL of 50 mM potassium phosphate		
Number of assays: Each kit contains sufficient reagents for five 96-well plates at 6 mL per plate based on the protocol below, with ~20% excess for pipetting considerations or for partial-plate assays.			
Approximate fluorescence excitation/emission maxima: Amplex® UltraRed reagent: ~568/581 in nm.			
* 1 unit = the amount of enzyme that will convert maltose, in the presence of inorganic phosphate, to 1.0 µmole of D-glucose and D-glucose 1-phosphate per minute at 37°C, pH 7.0.			
† 1 unit = the amount of enzyme that will oxidize 1.0 µmole of β-D-glucose to D-gluconolactone and H ₂ O ₂ per minute at 35°C, pH 5.1.			
‡ 1 unit = the amount of enzyme that will form 1.0 mg purpurogallin from pyrogallol in 20 seconds at 20°C, pH 6.0.			

Introduction

Phytases catalyze the sequential hydrolysis of phytate (myoinositol hexakis phosphate; phytin; phytic acid) to less phosphorylated myo-inositol compounds and inorganic phosphate. This kit detects phytase activity based on measurement of the phosphate released from the substrate phytic acid.

The EnzChek® Ultra Phytase Assay Kit utilizes a series of linked enzymatic reactions for the detection of phytase activity (Figure 1). In the initial step, phytase catalyzes the release of inorganic phosphate from phytic acid; in the presence of P_i , maltose phosphorylase converts maltose to glucose 1-phosphate and glucose. Glucose oxidase then converts the glucose to gluconolactone and H_2O_2 . Finally, with horseradish peroxidase (HRP) as a catalyst, the H_2O_2 reacts with the Amplex® UltraRed reagent to generate a fluorescent product, which has absorption and fluorescent emission maxima of ~568 nm and 581 nm, respectively (Figure 2). The resulting increase in fluorescence is proportional to the amount of P_i in the sample. This relative measure of phytase activity can be used as the basis of a standard curve of phytase enzyme standards with known activity.

The assay takes one hour at 37° C; under these conditions, the limit of detection is 0.001 FTU/mL and the intra-assay CV is ≤10%. The signal is not linear but can cover a two-log concentration range for phytase (Figure 3).

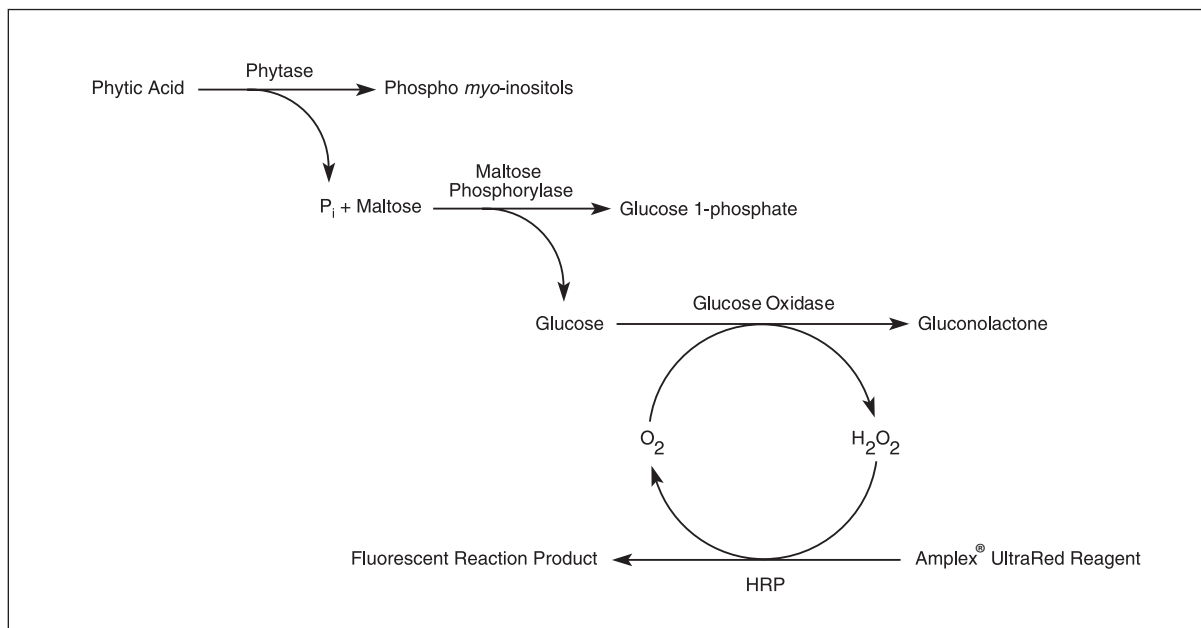


Figure 1. Principle of EnzChek® phytase assay.

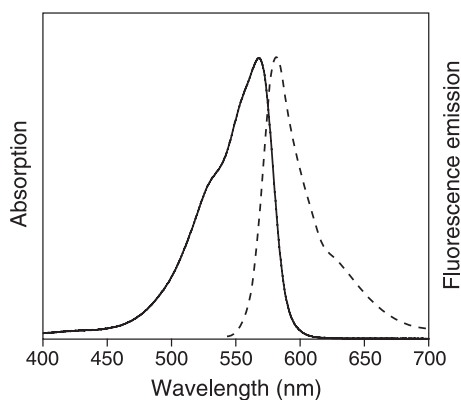


Figure 2. Normalized absorption and fluorescence emission spectra for the Amplex® UltraRed peroxidation product.

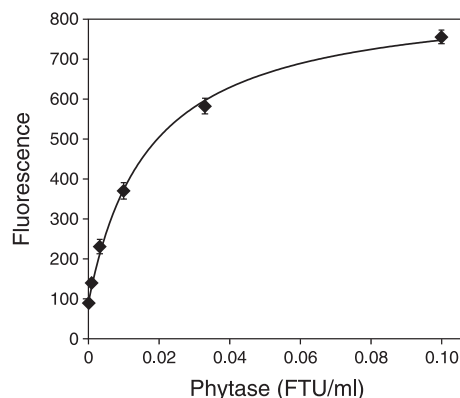


Figure 3. Assay of a standard phytase preparation using the EnzChek® Ultra phytase assay. A dilution series of Natuphos® 10000 L phytase (BASF) in 0.1 M acetate buffer (pH 5.5) was assayed with the EnzChek® Ultra Phytase Assay Kit. Incubation time was 60 minutes; phytase activity is shown in FTU/mL (FTU= phytase unit, as defined by the manufacturer). The data can be fit to a hyperbolic equation.

Before Starting

Materials Required But Not Provided

- Deionized water (dH₂O)

Caution

DMSO is known to facilitate the entry of organic molecules into tissues. Handle reagents containing DMSO (e.g., Amplex® UltraRed reagent stock solution in DMSO) using equipment and practices appropriate for the hazards posed by such materials. Dispose of the reagents in compliance with all pertaining local regulations.

Preparing Solutions

10 mM Amplex® UltraRed reagent stock solution

- 1.1 To prepare a 10 mM stock solution of Amplex® UltraRed reagent, allow one vial of Amplex® UltraRed reagent (Component A) and the DMSO (Component B) to warm to room temperature. Immediately prior to use, dissolve the contents of the vial of Amplex® UltraRed reagent in 60 µL DMSO. Each vial of Amplex® UltraRed reagent is sufficient for approximately 120 assays, with a final reaction volume of 100 µL per assay. Store the stock solution frozen at ≤−20°C, protected from light.

1X Reaction Buffer working solution

- 1.2 To prepare a 1X working solution of Reaction Buffer (0.1 M sodium acetate, pH 5.5), add 2.5 mL of 10X Reaction Buffer stock solution (Component C) to 22.5 mL of deionized water (dH₂O). This 25 mL volume of 1X Reaction Buffer is sufficient to formulate the stock solutions and dilute the reaction buffer, enzymes, and standards.

40 mM Phytic Acid

- 1.3 To prepare a 40 mM solution of phytic acid, dissolve the contents of the vial of phytic acid (Component D) in 1.5 mL of 1X Reaction Buffer. Store any remaining phytic acid solution frozen at ≤−20°C.

200 U/mL maltose phosphorylase stock solution

- 1.4 To prepare a 200 U/mL stock solution of maltose phosphorylase, dissolve the contents of the vial of maltose phosphorylase (Component E) in 750 µL of 1X Reaction Buffer. Store any remaining maltose phosphorylase solution frozen at ≤−20°C.

200 U/mL glucose oxidase stock solution

- 1.5 To prepare a 200 U/mL solution of glucose oxidase, dissolve the contents of the vial of glucose oxidase (Component F) in 1.0 mL of 1X Reaction Buffer. Store any remaining glucose oxidase solution frozen at ≤−20°C.

100 U/mL horseradish peroxidase (HRP) stock solution

- 1.6 To prepare a 100 U/mL stock solution of horseradish peroxidase (HRP), dissolve the contents of the vial of HRP (Component G) in 300 µL of 1X Reaction Buffer. Store any remaining HRP solution frozen at ≤−20°C.

40 mM maltose stock solution

- 1.7 To prepare a 40 mM stock solution of maltose, dissolve the contents of the vial of maltose (Component H) in 1.39 mL of 1X Reaction Buffer. Store any remaining maltose solution frozen at ≤−20°C.

Experimental Protocols

General Considerations The dependence of this assay on phytic acid as a phosphate source is diagnostic for phytase activity in an unknown mixture. The phytic acid substrate provided in this kit is of the highest quality commonly available; nevertheless, at 1 mM phytic acid, we observe 10–20 μ M phosphate-equivalent background.

The EnzChek® phytase assay utilizes a linked-enzyme system for the accurate detection of free phosphate. Contaminants that alter the activity of the enzymes in this assay system can interfere with correct phytase activity determination; common interfering agents may include phosphate, glucose, and reducing agents such as DTT. In the presence of a high excess of phosphate, the Amplex® UltraRed reagent can be further oxidized to a nonfluorescent state, resulting in a decrease in the observed signal and an inaccurate assessment of phytase activity.

Phytase Activity Assay

- 2.1 Prepare a standard curve by diluting a sample of known phytase activity in 1X Reaction Buffer (prepared in step 1.2) to a final range of 0.001 to 0.1 FTU/mL, with 1X buffer as a negative (no enzyme) control. A volume of 50 μ L will be used for each reaction.
- 2.2 Prepare a dilution series of the unknown phytase sample sufficient to span the activity range covered by the standard curve.
- 2.3 If desired, prepare a positive control for phosphate detection by diluting the 50 mM phosphate standard (Component I) to 1–100 μ M (20 μ M works well) in 1X Reaction Buffer. The effective phosphate concentration range for this kit is 1–200 μ M.
- 2.4 Pipet 50 μ L of the diluted samples and controls into separate wells of a microplate.
- 2.5 Prepare a working reaction mixture of 100 μ M Amplex® UltraRed reagent containing 4 U/mL maltose phosphorylase, 0.4 mM maltose, 2 U/mL glucose oxidase, 0.4 U/mL HRP and 2 mM phytic acid.

Note: A 96-well plate generally requires at least 10% excess (5.5 mL). To prepare 3, 4, 5, or 6 mL of this reaction mixture (as needed), refer to Table 1. Prepare only the amount needed for the experiment at hand; use the reaction mixture within 5 minutes of preparation.

Table 2. Guide to preparing the reaction mixture.

Volume of Reaction Mixture Needed	Use						
	1X Reaction Buffer	40 mM Phytic Acid	10 mM Amplex® UltraRed Reagent	Maltose Phosphorylase	Glucose Oxidase	Horseradish Peroxidase	Maltose
3 mL	2.7 mL	150 μ L	30 μ L	60 μ L	30 μ L	12 μ L	30 μ L
4 mL	3.6 mL	200 μ L	40 μ L	80 μ L	40 μ L	16 μ L	40 μ L
5 mL	4.5 mL	250 μ L	50 μ L	100 μ L	50 μ L	20 μ L	50 μ L
6 mL	5.4 mL	300 μ L	60 μ L	120 μ L	60 μ L	24 μ L	60 μ L

- 2.6 Begin the reactions by adding 50 μ L of the reaction mixture to each microplate well containing the samples and controls.

2.7 Incubate the reactions for 60 minutes at 37°C, **protected from light**.

Note: Since the assay reaction is continuous (not terminated), you may measure the fluorescence at multiple time points to determine the rate of fluorescence production, if desired.

2.8 Measure sample fluorescence with a fluorescence microplate reader set for excitation in the range of 530–560 nm and emission detection at 580–590 nm (Figure 2).

2.9 You can obtain the phytase activity for unknown samples by using a standard curve generated from assaying samples of a phytase standard of known activity (see step 2.1). Phytase activity data generated from the EnzChek® Ultra Phytase Assay are best described by a hyperbolic curve; you can determine unknown phytase activities mathematically, using the equation for the hyperbolic curve of best fit to the standard data. Alternatively, you can evaluate unknown phytase activities by direct comparison to the phytase standard curve.

Product List

Current prices may be obtained from our website or from our Customer Service Department.

Cat. no.	Product Name	Unit Size
E33701	EnzChek® Ultra Phytase Assay Kit *500 assays*	1 kit

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