QuantiChrom™ Phytase Assay Kit (DPHT-100)

Quantitative Colorimetric Determination of Phytase Activity at 620nm

DESCRIPTION

PHYTASE catalyzes the hydrolysis of phosphoester bonds on myoinositol-(1,2,3,4,5,6)-hexakisphosphate (Phytic acid or IP_6) thereby releasing inositol and phosphate. Phytic acid is a major storage reservoir for phosphate in plants. Phytase is abundant in grains such as wheat and barley, and the hydrolysis of phytic acid by single-stomached animals is a crucial aspect of animal nutrition. In addition, the lack of phytase in single-stomached animals can lead to excessive phosphorus leaching into the environment due to undigested phytic acid.

Simple, direct and automation-ready procedures for measuring phytase activity are very desirable. BioAssay Systems' QuantiChrom™ Phytase Assay is based on our Malachite Green Phosphate Assay (POMG-25H). The color intensity, measured at 620 nm, is proportionate to the amount of phosphate released from phytic acid.

KEY FEATURES

Simple. No complex detection reagents to mix.
High sensitivity and wide detection range: Detection range of 0.01 to 20 U/L phytase in a 96-well plate assay.
Fast and convenient: Homogeneous "mix-and-measure" assay allows for quantification of phytase activity within 60 minutes.

APPLICATIONS

Direct assays of phytase activity in agricultural and biological samples.

KIT CONTENTS (100 TESTS IN 96-WELL PLATES)

Assay Buffer (pH 5.5):	10 mL	**POMG Reagent A:**	2.5 mL
Phytic Acid:	120 µL	**POMG Reagent B:**	120 µL
Standard:	120 µL 1 mM Phosphate		

Storage Conditions. The kit is shipped at room temperature. Store all components at 4°C upon receiving. Shelf life: 12 months after receipt.

Precautions: Reagents are for research use only. Normal precautions for laboratory reagents should be exercised while using the reagents. Please refer to Material Safety Data Sheet for detailed information.

PROCEDURES

Sample Preparation. Phosphate-containing detergents and buffers must be avoided in the preparation of phytase extracts as the assay is extremely sensitive to free phosphate. If the sample contains phosphate, we recommend removing it by at least 3 washes with a 10kDa NMWL membrane filter (e.g. Amicon® Ultra 0.5 mL Ultracel or similar).

Working Reagent Preparation. Each well requires 75 µL of Phytic Acid Working Reagent (*WR*) per assay well. Prepare enough *WR* by mixing 1 µL of the Phytic acid stock with 80 µL of the Assay Buffer per assay well.

Color Development Reagent Preparation. Each well requires 20 µL Color Development Reagent. Prepare enough Color Development Reagent by mixing 100 vol of POMG Reagent A with 1 vol of POMG Reagent B (e.g. 1 mL of Reagent A and 10 µL of Reagent B). Working Reagent is stable for at least 1 day at room temperature.

Important*: 1. All reagents must be brought to room temperature before use. 2. Before each assay, it is important to check that all enzyme preparations and assay buffers do not contain free phosphate. This can be conveniently done by adding 20 µL of the Color Development Reagent to 80 µL of sample solution. The blank OD values at 620 nm should be lower than 0.2 at 30min. If the OD readings are higher than 0.2, check the phosphate level of the water. 3. Precipitation may occur at high concentrations of phosphate (>100 µM), proteins, and metals. If precipitation occurs, perform a serial dilution of the sample in H_2O, run the assay and choose the dilution factor based on the wells with no precipitation. Repeat the assay using the appropriately diluted samples. 4. For an enzyme sample of unknown activity, it is prudent to run a serial dilution in assay buffer to bring the activity within the detection range. High protein concentrations can interfere with the Color Development through precipitation.*

1. Pipette 5 µL of phytase-containing extract into separate wells of a clear bottom 96-well plate. Reserve one well for a Blank (5 µL of buffer, no enzyme). Initiate the reaction by adding 75 µL of the *WR* into each well. Tap plate briefly to mix the reaction mixture. *Note: If the assay is to be performed at another temperature (e.g. 37°C), warm up the Working Reagent to this temperature prior to adding it to the sample.*
2. Incubate the plate at room temperature or the desired temperature for 30 minutes.
3. Phosphate Standard Preparation: Prepare a 40 µM phosphate premix solution by pipetting 20 µL of the 1 mM phosphate standard to 480 µL of distilled water. Number the tubes. Dilute standards as shown in the following Table. Pipette 80 µL of each standard in duplicate into separate wells of the assay plate.

No	Premix + H_2O	Final Vol (µL)	[Phosphate] (µM)
1	200µL + 0µL	200	40
2	120µL + 80µL	200	24
3	60µL + 140µL	200	12
4	0µL + 200µL	200	0

4. At 30 minutes, add 20 µL of the Color Development Reagent to each well. Mix gently by tapping the plate.
6. Incubate for an additional 30 min at room temperature for color development.
7. Measure absorbance at 600 nm - 660nm (peak: 620 nm) with a plate reader.

   Note: If the observed phosphate concentration is equal to or greater than the 40 µM Phosphate Standard, dilute the enzyme extract in the buffer and repeat the assay.
8. **Calculation:** The phytase activity in the sample is calculated as follows:

$$\text{Phytase Activity} = \frac{OD_{SAMPLE} - OD_{BLANK}}{Slope} \times \text{Reaction Vol (L)} / \text{time} / \text{Sample Vol (L)}$$

$$= \frac{OD_{SAMPLE} - OD_{BLANK}}{Slope} \times 80 \times 10^{-6}\ \text{L} / 30\text{min} / (5\times10^{-6}\ \text{L})$$

$$= \frac{OD_{SAMPLE} - OD_{BLANK}}{Slope} \times 0.533\ (\text{U/L})$$

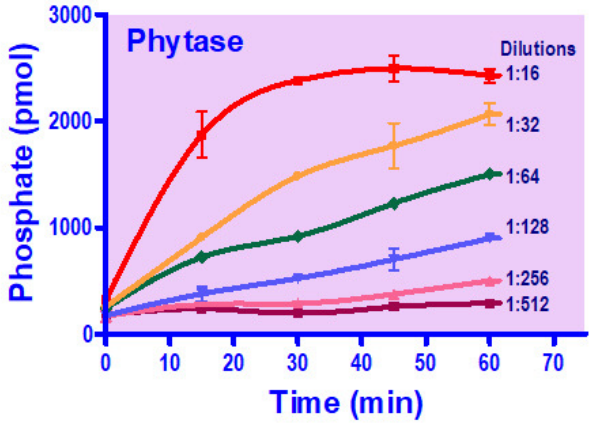
Where OD_{SAMPLE} and OD_{BLANK} are the sample and blank absorbances, respectively, and the Slope is the Slope (μM^{-1}) of the phosphate standard curve.

Unit definition: one unit (U) of enzyme catalyzes the release of 1 µmole of substrate per minute under the assay conditions (pH 5.5).

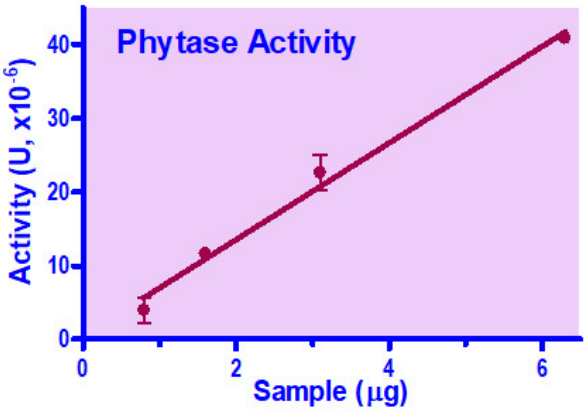
MATERIALS REQUIRED, BUT NOT PROVIDED

Pipeting (multi-channel) devices. Clear-bottom 96-well plates (e.g. Corning Costar) and plate reader. 10kDa NMWL membrane filters.

EXAMPLE. Fifty milligrams (50 mg) of crude wheat phytase (Sigma P1259) was resuspended in 1.25 mL of 50 mM Sodium Acetate buffer (pH5.5) with 1 mM $CaCl_2$ and mixed gently for 30 minutes at Room Temperature then transferred to 4ºC overnight. After overnight incubation, the extract was clarified by centrifugation at 14,000 rpm for 10 minutes. The supernatant was buffer exchanged three times using an Amicon® Ultra 0.5 mL Ultracel 10kDa NMWL filter and the Sodium Acetate buffer and brought back to the initial 1.25 mL volume. This Phytase extract was diluted in the Sodium Acetate buffer and assayed according to the standard protocol. The specific activity was calculated to be 6.57 ± 0.98 U/g sample.



Wheat phytase activity time course at room temperature. The reaction was carried out as described in the protocol with separate reactions in duplicate for each time point.



Wheat phytase activity dilution series at room temperature. A plot of the initial rate at 30 minutes against enzyme sample (µg/well) was made.

LITERATURE

1. Dionisio, G., et al (2011) Cloning and Characterization of Purple Acid Phosphatase Phytases from Wheat, Barley, Maize, and Rice. *Plant Physiol.* 156:1087.
2. Madsen, C. K. and Brinch-Pedersen, H. (2019) Molecular Advances on Phytases in Barley and Wheat. *Int. J. Mol. Sci.* 20:2459
3. Nakano, T., et al (1999) Purification and Characterization of Phytase from Bran of *Triticum aestivum* L. cv. Nourin #61. *Food Sci. Technol. Res.* 5(1): 18.