Technical Bulletin

Phytase Assay Kit

Catalogue Number MAK616

Product Description

Phytase catalyzes the hydrolysis of phosphoester bonds on myoinositol-(1,2,3,4,5,6)-hexakisphosphate (Phytic acid or IP6) 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.

The color intensity, measured at 620 nm, is proportionate to the amount of phosphate released from phytic acid. The detection range is 0.01 to 20 U/L phytase in a 96-well plate assay.

Components

The kit is sufficient for 100 colorimetric assays in 96-well plates.

- Assay Buffer 10 mL
  Catalogue Number MAK616A
- Phytic Acid 120 µL
  Catalogue Number MAK616B
- POMG Reagent A 2.5 mL
  Catalogue Number MAK616C
- POMG Reagent B 120 µL
  Catalogue Number MAK616D
- Standard 120 µL
  Catalogue Number MAK616E

Reagents and Equipment Required but Not Provided

- Pipetting devices and accessories (e.g., multichannel pipettor)
- Spectrophotometric multiwell plate reader capable of reading at 600–660 nm.
- Clear plates for colorimetric assays (Catalogue number M2936 or equivalent) Cell culture or tissue culture treated plates are not recommended.
- 1.5 mL microcentrifuge tubes
- 10kDa NMWL membrane filters

Precautions and Disclaimer

For R&D use only. Not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Storage/Stability

The product is shipped at ambient temperature. Store kit at 4°C upon receipt.

Preparation Instructions

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

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

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). This Reagent is stable for at least 1 day at room temperature.

Procedure

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 30 minutes. 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 Color Development through precipitation.

Assay Reaction

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.

Phosphate Standard Preparation:

3. Prepare a 40 µM phosphate premix solution by pipetting 20 µL of the 1 mM phosphate Standard (MAK616E) to 480 µL of distilled water. Number the tubes. Dilute standards as shown in the following Table 1.

Table 1.
Standard Preparation

Std #	Premix (µL)	Purified water (µL)	Conc. (µM)
1	200	0	40
2	120	80	24
3	60	140	12
4	0	200	0

4. Pipette 80 µL of each standard in duplicate into separate wells of the assay plate.
5. 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.

Results

Calculations

The phytase activity in the sample is calculated as follows:

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

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

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

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).

Figure 1.
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.

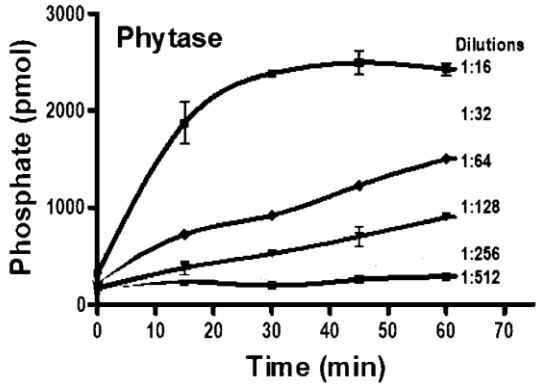
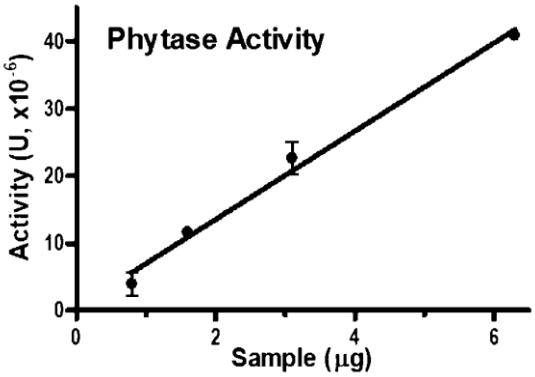


Figure 2.
Wheat phytase activity dilution series at room temperature. A plot of the initial rate at 30 minutes against enzyme sample (µg/well) was made.



References

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.

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mak616pis Rev 02/26