CytoPhos Phosphate Assay BIOCHEM KIT™

Cat. # BK054

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Manual Contents

I: Introduction

ATPase End Point Assay

The ATPase end-point assay is an extremely quick and economical way to measure inorganic phosphate (Pi) generated during the enzymatic hydrolysis of ATP. Large numbers of assays can be performed simultaneously in a homogenous reaction, making the assay highly suitable for high throughput screen (HTS) applications.

The ATPase end-point assay is an adaptation of the method of Kodama et al. Biochem. 1986. 99: 1465-1472.

II: Kit Contents: Reconstitution and Storage

This kit contains enough reagents for approximately 960 assays of 100 µl volume. Many of the kit components are provided in lyophilized form. Prior to beginning the assay, you will need to reconstitute components as shown in Table 1. When properly stored and reconstituted, components are guaranteed stable for 6 months.

Table 1: Kit Contents, storage and reconstitution.

Kit Component	Cat. # /Part # (Quantity)	Reconstitution	Storage after reconstitution
CytoPhos Reagent	Part # CYPH	One bottle, 70 ml reagent. No reconstitution necessary	4°C
Phosphate Standard	Part# PI01-L	One tube, lyophilized Reconstitute in 1ml of phosphate free deionized water Contains 1ml of 0.1 mM phosphate standard after reconstitution	4°C

* Items with part numbers (Part #) are not sold separately and available only in kit format. Items with catalog numbers (Cat. #) are available separately.

III: Important Technical Notes

The following technical notes should be carefully read prior to beginning the assay.

Instrument requirements
The ATPase end-point assay is based upon a colorimetric change, measured at 650 nm. A spectrophotometer capable of measuring this wavelength will be required.
We recommend the use of a SpectraMAX250 Microplate Spectrophotometer System (Molecular Devices). This machine uses a monochromatic light source and is one the more sensitive machines available. An example of the required settings is given below:

Instrument settings for SpectraMAX250

Spectrophotometer Parameter	Setting
Measurement type	End-point
Absorbance wavelength	650 nm
Temperature	Room temp.
Shaking	5 s medium, orbital

Reagents and Reaction Conditions
This assay is not compatible with phosphate containing buffers. Proteins that are present in a phosphate buffer must be dialyzed or prepared in an alternative buffer system such as Tris. The ATPase assay is sensitive over a range of 1 µM – 15 µM Pi (equivalent to 0.1 nmoles – 1.5 nmoles Pi in 100 µl reaction volume) and can be performed over a pH range of 6.5 – 8.5.

Assay Optimization
The ATPase end-point assay kit has been developed to provide a good general substrate for a broad range of ATPase proteins, however, there are several parameters that may affect ATPase activity, and these include:

Protein concentration A titration of the ATPase of interest should be performed to achieve optimal results. This assay is suitable for protein concentrations below 1 mg/ml. If higher protein concentrations are required, the PhosFree Phosphatase Assay Kit (Cat. # BK050) should be considered.

Reaction buffer conditions Salt concentration (eg. 20 mM-1000 mM) and pH should be titrated for optimal activity.

ATP concentration To minimize background readings an ATP concentration of 0.3 - 0.6 mM is recommended. An ATP titration should be performed to obtain optimal results.

Control Reactions It is important to include control reactions in the assay, particularly if the ATPase of interest is in an impure state.

Half Area Well Plates We recommend the use of a half area well plate (180 µl volume) to perform the assays, as this will maintain the pathlength while allowing smaller reaction volumes to be used. The protocols for this assay will describe reactions for a 100 µl final volume. If standard 300 µl volume wells are to be used, we recommend using a 200 µl final volume.

IV: Phosphate Standard Curve

A standard curve can be generated for inorganic phosphate (Pi) using the phosphate standard supplied in this kit. The linear range extends from approximately 0.1 nmoles to 1.5 nmoles of Pi. Each microliter of the phosphate standard is equivalent to 0.1 nmoles of Pi.

Method

Add the reagents shown in Table 1 to the wells of a half area 96 well plate and incubate for 10 min at room temperature.
Set the spectrophotometer to read an end-point assay at absorbance 650 nm.

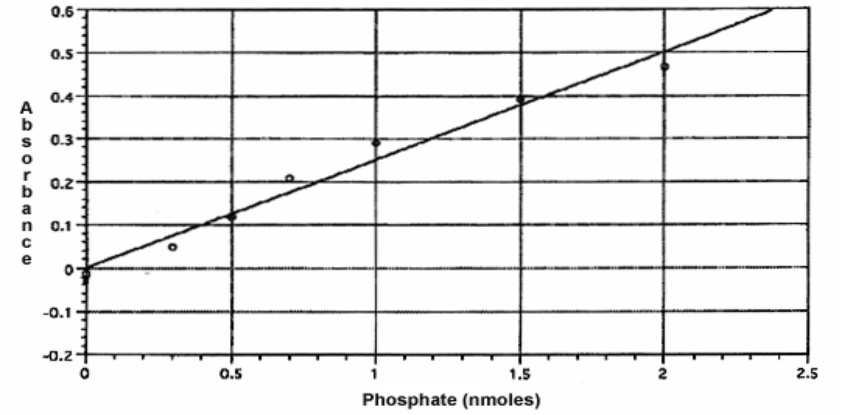
Designate well A1 as the BLANK and read the samples

Plot a phosphate standard curve from your results (see Figure 1).

Table 1: Phosphate Standard Curve Reactions

Well	Phosphate free distilled water (µl)	0.1 mM Phosphate Standard (µl)	CytoPhos Reagent (µl)	n moles of Pi per well
A1 (Blank)	30	0	70	0
B1	30	0	70	0
C1	27	3	70	0.3
D1	25	5	70	0.5
E1	23	7	70	0.7
F1	20	10	70	1.0
G1	15	15	70	1.5
H1	10	20	70	2.0

Figure 1: Typical Phosphate Standard Curve



V: ATPase Assay Protocol

The ATPase end-point assay measures inorganic phosphate (Pi) generation, therefore, it is necessary that clean Pi free assay plates be used. It is strongly recommended to use a half area well plate to increase the absorbance signal. If a standard 96 well plate is used (300 µl well volume) we recommend using a 200 µl final volume.

Reaction Blank

Each experimental setup should contain a blank sample that consists of the reaction buffer plus ATP only.

Reaction Volumes

Each assay should have a final volume of 100 µl; the ATPase reaction should have a volume of 30 µl to which you will add 70 µl of CytoPhos reagent for developing the reaction. The reaction volumes can be scaled up as required.

The relative volume of CytoPhos reagent to reaction buffer should be titrated to ensure that the CytoPhos reagent is not saturated.

Starting the Reaction

The ATPase activity is started by the addition of ATP. It is therefore highly advisable to add ATP using a multichannel pipet. In this way all reaction will begin simultaneously.

Terminating and Reading the Reactions

1. Terminate the reactions by adding 70 µl of CytoPhos to each well.
2. Incubate the plate at room temperature for 10 min.
3. Designate the appropriate “blank” samples
4. Read the absorbance at 650 nm.

Interpretation of Experimental Results

The phosphate standard curve generated from Section IV can be then used to estimate the amount of Pi generated in the ATPase reactions.

VI: References and Citations

Reference

1. Kodama, T. et al. J. (1986) Biochem. 99: 1465-1472

Product Citations/Related Products

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