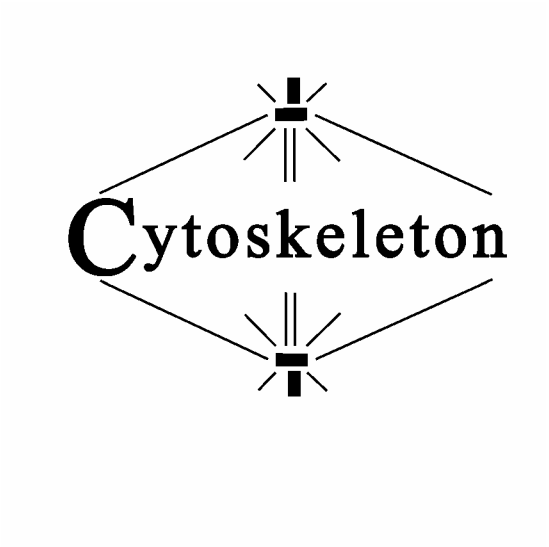


CytoPhos Phosphate Assay
BIOCHEM KIT

BK054

ORDERING INFORMATION

To order by phone: (303) - 322 - 2254
To order by Fax: (303) - 322 - 2257
Technical assistance: (303) - 322 – 2254 or tservice@cytoskeleton.com
World Wide Web: www.cytoskeleton.com
Write to us: Cytoskeleton, Inc., 1830 S. Acoma Street, Denver, CO 80223. U.S.A.

MANUAL CONTENTS

Section I	Introduction ……..	3
Section II	Important Technical Notes ……..	3
Section III	Kit Contents ……	4
Section IV	Standard Phosphate Curve ……..	5
Section V	ATPase Protocol …….	6
Section VII	References …….	6
Section VIII	Related Products ……	6

Section I: ATPase or GTPase End Point Assay Introduction

The ATPase end-point assay (or GTPase) 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 HTS applications. The assay is an adaptation of the method of Kodama et al (1).

Section II: Important Technical Notes

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

Reagents and Reaction Conditions

1) This assay is not compatible with phosphate containing buffers. If your ATPase protein is in a phosphate buffer you must remove this by dialysis or by preparing the protein in an alternative buffer such as Tris.
2) The reaction is sensitive over a range of 1 uM – 15 uM Pi (equivalent to 0.1 nmoles – 1.5 nmoles Pi in 100 ul 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.
There are several parameters that may particularly affect ATPae activity, these include;

- **Protein concentration**. A titiration of the ATPase of interest should be performed to achieve optimal results. This assay is suitable for protein concentrations below 1mg/ml, if higher concentrations are used then BK050 should be used.
- **Reaction buffer conditions**. In particular pH and salt concentration 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 your ATPase of interest is in an impure state.
- **Half Area Well Plates** If using a 96 well plate for this assay, we strongly recommend using a half area well plate (180 ul volume) to perform the assays as this will maintain pathlength while allowing smaller reaction volumes to be used. The assay protocol will describe reactions of 100 ul final volume. If standard 300 ul volume wells are used, you will get approximately 50% reduction in absolute values per 100 ul reaction.

Instrumentation

The assay is based upon a colorimetric change, measured at 650 nm you will require a spectrophotometer capable of measuring at this wavelength.

Section III: Kit Contents

This kit contains sufficient reagents for approximately 960 assays of 100 ul volume.

KIT COMPONENT	DESCRIPTION
CytoPhos Reagent	One bottle. 70 mls.
Phosphate standard	One tube. Contains 1 ml of 0.1 mM phosphate standard.

Section IV: Standard Phosphate 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 n moles to 1.5 n moles of Pi. Each ul of the supplied phosphate standard is equivalent to 0.1 n moles of Pi.

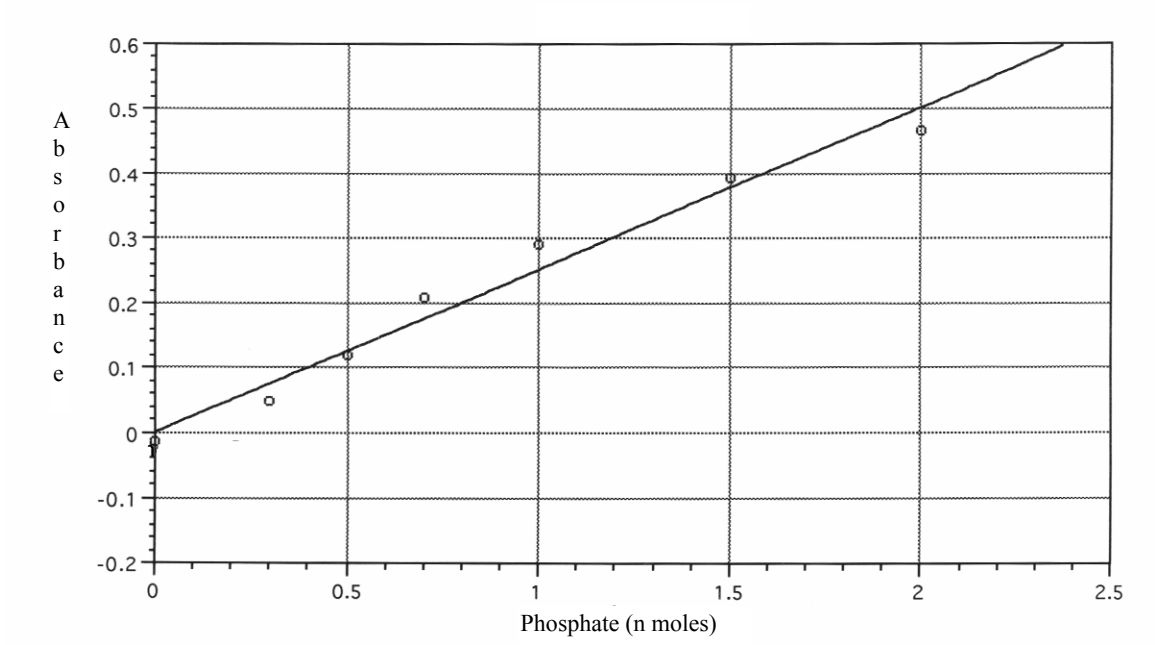
Method

1) Add the reagents shown in Table 1 to the wells of a half area 96 well plate and incubate for 10 minutes at room temperature.
2) Set the spectrophotometer to read an end-point assay at absorbance 650 nm.
3) Designate well A1 as the BLANK and read the plate.
4) Plot a Phosphate Standard curve from your results (see Figure 1).

Table 2: Standard Pi Curve Reactions

Well Allocation	Pi free distilled water (ul)	0.1 mM Phosphate Standard (ul)	CytoPhos Reagent (ul)	n moles of Pi per well
A1	30	zero	70	BLANK
B1	30	zero	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: Standard Pi Curve Generated From The Above Reactions



Section V: Performing The ATPase Reactions

Instrumentation Settings and Microtiter Plates

The reaction is based upon an absorbance reading at 650nm. Your spectrophotometer should therefore be set at an absorbance wavelength of 650 nm for readings. The spectrophotometer should be at room temperature and set on end-point reading mode.

As this assay measures inorganic phosphate (Pi) generation, one needs to make sure that clean Pi free plates are used. Always include a negative control reaction that contains all reaction components minus the kinesin under study. This assay is set up as a 100 ul final volume and it is strongly recommended to use a half area well plate to increase absorbance signal. If a 300 ul well volume is used, the readings will be approximately half that of the half area well readings for 100 ul final volume.

Reaction Blank

Consists of Reaction Buffer plus ATP only.

Reaction Volumes

For a final volume of 100 ul make the ATPase reaction 30 ul and add 70 ul of CytoPhos reagent for developing the reaction. The reaction volumes can be scaled up as required. Also the relative volume of CytoPhos to reaction should be titrated to make sure you are not saturating the CytoPhos reagent.

Starting the Reaction

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

Note: BEFORE ADDING THE ATP make sure that the specrophotometer is set up correctly in end point read mode at 650 nm wavelength.

Terminating and Reading the Reactions

1) Terminate the reactions by adding 70 ul of CytoPhos to each well.
2) Allow this to react for 10 minutes and take readings at 650 nm. Designate the reaction in well A1 as the blank.

Interpretation of Experimental Results

The standard Pi curve can be used to estimate the amount of Pi generated in the kinesin ATPase reactions, which is then converted into nmoles of Pi per min per mg of enzyme (not total protein).

Section VI: References

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

Section VII: Related Products

BK050	PhosFree Phosphate Assay	For high protein concentration reaction solutions.
BK051	ELIPA ATPase Kinetic Assay	Realtime kinetic mid-range sensitivity assay.
BK055	EasyRad Phosphate Assay	Highly sensitive for Small G-proteins or G-protein coupled receptors