V.8.0

KINESIN ATPase END-POINT BIOCHEM KIT

Cat. # BK053

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Section I: Kinesin End Point Assay Introduction

Eukaryotic kinesin motor proteins orchestrate a wide range of kinetic events within a cell. They have been shown to move cargoes, such as chromosomes and vesicles, along microtubule tracks (1). They also play a major role in the organization of cytoskeletal architecture as evidenced in the establishment of the microtubule spindle during mitosis (2).

Kinesins operate by utilizing the energy of ATP hydrolysis to move along their microtubule (MT) substrates. Once a kinesin motor binds to its MT track, the ATPase rate of the motor is often enhanced several hundred to several thousand fold (3). MT activated kinesin ATPase is a major parameter in motor function and serves as a powerful method to monitor / study kinesin activity under various experimental conditions.

As part of its **C**ytoskeleton **M**otor **W**erks (CMW) line of research reagents, Cytoskeleton Inc. has developed the Kinesin ATPase End-Point Biochem Kit (Cat. # BK053). The kinesin end-point assay is an extremely quick and economical way to measure inorganic phosphate (Pi) generated during the microtubule activated ATPase activity of kinesin motor proteins. 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 (4).

More detailed kinetic studies for kinesin MT activated ATPase assays can be performed with Cytoskeleton Motor Werks Kit Cat. # BK060. Cytoskeleton Inc. also offers a wide range of kinesin motors (see Section VIII).

II: Important Technical Notes

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

Reagents and Reaction Conditions

1) This kit contains sufficient microtubules (MTs), reaction buffers and Pi detection reagent (CytoPhos) to carry out approximately 960 standard reactions (100 µl volume). The kinesin heavy chain motor domain protein included in this kit as a control is sufficient for approximately 240 reactions. A list of other kinesin proteins available from Cytoskeleton Inc. can be found in Section VIII.

2) Many of the reagents in this kit require reconstitution and aliquoting into convenient experiment sized amounts. It is important to carry out the aliquoting step as freeze / thawing of some reagents (for example kinesin protein and microtubules) is not recommended as it will result in inactivation of the reagents.

3) ATP is not included in this kit as ATP is not compatible with storage at 4°C for this protocol; the ATP product (Cat. # BSA04**)** from Cytoskeleton **IS NOT SUITABLE** for this assay. ATP can be purchased from several vendors including Sigma (Cat. # A3377). The ATP should be made to 100 mM pH 7.0 in a non phosphate containing buffer such as 100 mM PIPES pH 7.0 and snap frozen in 20 µl volumes. The quality of the ATP is critical for low background signal in this assay so AVOID REPEATED FREEZE / THAWS of this reagent. ATP powder should be stored at –20°C.

4) This assay is not compatible with phosphate containing buffers. If your kinesin protein is in a phosphate buffer you must remove this by dialysis or by preparing the protein in an alternative buffer such as Tris.

5) The reaction is sensitive over a range of 2 µM – 15 µM Pi (equivalent to 0.2 nmoles – 1.5 nmoles Pi in 100 µl reaction volume) and can be performed over a pH range of 6.5 – 8.5.

6) Temperature is very important for this assay. Microtubules (MTs) will depolymerize even in taxol if placed on ice. Take note when it is indicated to place a solution at **room temperature**.

Assay Optimization

The Kinesin ATPase end-point assay kit has been developed to provide a good general substrate for a broad range of kinesin proteins. The control protein, kinesin heavy chain motor domain, should give a rate of 1200 – 1500 ATPs hydrolyzed per minute per mg of protein under the reaction conditions described in Section VI. These values are comparable to published data. It should be noted, however, that optimization of the end point assay may be needed for any given kinesin protein.

There are several parameters that may particularly affect a kinesins MT activated ATPase activity, these include:

- **Protein concentration**. A titration of the kinesin of interest should be performed to achieve optimal results. Kinesin protein titration is described in the Assay Protocol in Section VI.
- **Reaction buffer conditions**. In particular pH and salt concentration should be titrated for optimal activity. In general final salt concentrations should be kept below 20 mM.
- **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. ATP must be purchased separately and made up to 100 mM stock.
- **Control Reactions**. It is important to include control reactions in the assay, particularly if your kinesin of interest is in an impure state. Control reactions are discussed in Section VI.
- **Half Area Well Plates** If using a 96 well plate for this assay, we strongly recommend using a half area well plate (180 µl volume) to perform the assays as this will maintain path length while allowing smaller reaction volumes to be used. The assay protocol will describe reactions of 100 µl final volume. If standard 300 µl volume wells are used, you will get approximately 50% reduction in absolute values per 100 µl 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.

III: Kit Contents

This kit contains sufficient reagents for approximately 960 assays of 100 µl volume and sufficient Kinesin heavy chain control protein for approximately 240 assays.

KIT COMPONENT	DESCRIPTION
CytoPhos Reagent (**Part # CYPH**)	One bottle. 70 ml.
Phosphate standard (**Part # PI01-L**)	One tube, lyophilized. Contains 1 ml of 0.1 mM phosphate standard when rehydrated.
Kinesin Motor Protein (**Cat. # KR01**)	2 tubes of kinesin motor domain-GST tagged protein, lyophilized.
Pre-formed Microtubules (**Cat. # MT002**)	4 tubes, lyophilized.
Kinesin Reaction Buffer (**Part # KRB01**)	1 bottle. 100 ml.
Taxol (**Cat.# TXD01**)	4 tubes, lyophilized.
DMSO (**Part # DMSO**)	1 tube, used to resuspend taxol.
Labels for Aliquoted Reagents	Microtubules (MTs) need to be aliquoted into 40 individual tubes. The kit box supplied can be used as storage container for these aliquots and the label provided can be used to identify these frozen stocks. See Section VI for details. The label is "BK053 MT STOCKS". A label is also supplied for ATP stocks. The ATP is not provided in this kit, it is recommended to purchase ATP from Sigma (Cat. # A3377).

IV: Things to do Prior to Beginning the Assay

Prior to beginning the assay you will need to reconstitute several components as follows:

Kit Component	Reconstitution	Storage Conditions
Microtubules (Cat. # MT002)	1) Remove all components from the kit and use the provided label "BK053 MT STOCKS" to label the box. This will serve as a storage box for the MT aliquots that you will make. 2) Thaw one tube of Taxol stock and store at room temperature. 3) Aliquot 500 µl of **Kinesin Reaction Buffer** into an eppendorf tube and warm in a 37°C water bath for 10 minutes. 4) Supplement the Kinesin Reaction Buffer with 5 µl of Taxol stock and mix well. **Keep at room temperature**. 5) Add 100 µl of the taxol supplemented buffer to each tube of MTs and leave at **room temperature** for 10 minutes. 6) Carefully resuspend the MTs and pool the contents of all 4 tubes. 7) Pipette again to make sure that the MTs are completely resuspended. 8) Aliquot into 40 x 10 µl volumes and snap freeze in liquid nitrogen. NOTE: FREEZING IN LIQUID NITROGEN IS ESSENTIAL TO MAINTAIN THE INTEGRITY OF THE MTs. 9) Store MT stocks at -70°C.	Store frozen MTs at -70°C. Stable for > 6 months. Store reconstituted MTs at room temperature. Stable for 2-3 days under these conditions.
Taxol (Cat. # TXD01)	Reconstitute each tube with 100 µl of DMSO. This solution will freeze if placed on ice so always use at room temperature.	Store at -70°C. Stable for >6 months.
Kinesin Motor Control Protein (Cat. # KR01)	1) Reconstitute each tube of kinesin (Cat. # KR01) in 10 µl of 4°C Kinesin Reaction Buffer. This gives a stock of 2.5 mg/ml protein. 2) Aliquot into 10 x 2 µl volumes and snap freeze in liquid nitrogen. 3) Store kinesin stocks at -70°C.	Store at -70°C Stable for > 6 months.
CytoPhos Reagent (Part # CYPH)	No reconstitution required. It is recommended to aliquot this reagent into 7 x 10 ml aliquots. The reagent is highly sensitive to Pi and once contaminated it will be useless.	Store at room temperature Stable for >1 year.
Phosphate Standard (Part # PI01-L)	Resuspend in 1 ml of phosphate-free, deionized water.	Store at 4°C. Stable for 6 months
Kinesin Reaction Buffer (Part # KRB01)	No reconstitution required. It is recommended to aliquot this reagent into 10 x 10 ml to prevent inadvertent contamination of the whole stock with Pi.	Store at 4°C Stable for > 1 year.

ATP Stocks

ATP must be provided by the researcher, we recommend ATP from Sigma (Cat. # A3377).

Good quality ATP is essential to give a good signal to noise ratio in this assay. ATP powder stocks must be stored at –20°C or lower and the 100 mM stocks required for this assay should be made fresh and aliquoted into 20 µl volumes for future use. The 100 mM ATP stock can be made as follows:

Resuspend 55.1 mg of ATP disodium salt (551 g/mol) in 900 µl of 100 mM PIPES buffer pH 7.0 (any non phosphate containing buffer pH 7.0 can be used). Check the pH of the solution and pH to 7.0 with 5 M NaOH if necessary, make up the final volume to 1 ml with buffer. Aliquot into 50 x 20 µl volumes and store these at -20 to -70°C. A label is supplied to identify the ATP stocks aliquots.

V: 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 nmoles to 1.5 nmoles of Pi. Each microliter of the supplied phosphate standard is equivalent to 0.1 nmoles of Pi.

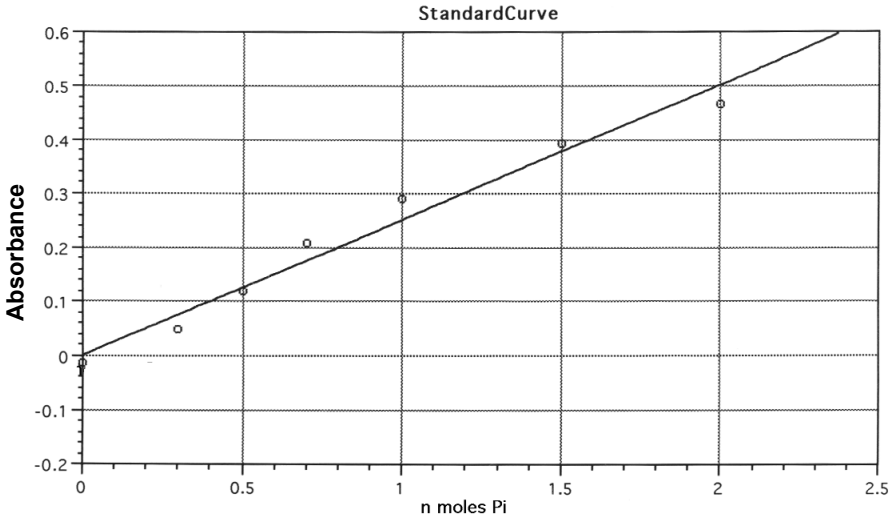
Method

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 (µl)	0.1 mM Phosphate Standard (µl)	CytoPhos Reagent (µl)	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



VI: Assay Protocol for 24 Assays

This protocol gives reagent volumes sufficient for 24 individual kinesin ATPase reactions. Eight of these reactions will be internal assay control reactions that include the kinesin heavy chain control protein provided in this kit (see **Table 2**). The remaining 16 reactions outline a simple kinesin protein titration for optimization of your kinesin of interest. Other optimization parameters are described in **Section II**.

If you wish to perform more than 24 reactions, simply scale up the reaction conditions shown below. Each kit contains sufficient reagents to carry out approximately 960 microtubule activated kinesin ATPase reactions. The kinesin heavy chain control protein included in this kit is sufficient for 240 individual assays. A list of other kinesin proteins available from Cytoskeleton Inc. can be found in **Section VIII**.

Instrumentation Settings and Microtiter Plates

The reaction is based upon an absorbance reading at 650 nm. 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 µl final volume and it is strongly recommended to use a half area well plate to increase absorbance signal. If a 300 µl well volume is used, the readings will be approximately half that of the half area well readings for 100 µl final volume.

Reaction Blank

Consists of Reaction Buffer plus ATP only (see **Table 2**).

Control Reactions

Control reactions are very important in this assay. The following controls should be performed each time an ATPase assay is run:

1) Reaction containing microtubules and ATP, minus kinesin protein (see **Table 2**).
2) Reaction containing kinesin protein of interest and ATP, minus microtubules (see **Table 2**).

Method

1) Aliquot 1 ml of Kinesin Reaction Buffer into an eppendorf and allow the buffer to warm to room temperature before moving to step 2.
2) Thaw one tube of taxol and supplement the reaction buffer with 10 µl of taxol. Keep both solutions at **ROOM TEMPERATURE**.
3) Thaw one 20 µl aliquot of ATP stock (100 mM ATP pH 7.0, ATP must be purchased separately, Sigma ATP [Cat. # A3377] is recommended, see section IV). NOTE: the ATP product from Cytoskeleton (**Cat. # BSA04 IS NOT SUITABLE FOR THIS ASSAY**). Dilute with 1 ml of ice cold water. Place on ice.
4) Thaw one tube of microtubules by placing in a room temperature water bath for 2-3 minutes. Store these at **ROOM TEMPERATURE**.
5) Add 240 µl of taxol supplemented reaction buffer to the microtubules to give a working stock of 0.2 mg/ml. Microtubules are stable at room temperature for several days. Diluted MTs should not be re-frozen.
6) Thaw one tube of kinesin (Cat. # KR01) and dilute with 58 µl of Reaction Buffer to give a working concentration of 0.08 µg/ul. Kinesin is stable at room temperature for several days. Diluted kinesin should not be re-frozen.
7) Perform reactions as outlined in **Table 2**. The first eight reactions act as controls for the assay. The remaining 16 reactions can be carried out using your kinesin of interest. There is sufficient kinesin heavy chain control protein in this kit to carry out approximately 240 kinesin ATPase reactions.

Table 2: MT Activated ATPase Reactions

Well	Reaction Buffer Plus Taxol (µl)	Microtubules (0.2 mg/ml) (µl)	Kinesin heavy chain Control Protein (0.08 mg/ml) (µl)	Your Kinesin of Interest **	Well Designation
A1	30	---------	---------	---------	Blank
B1	20	10	---------	---------	MT only Control
C1	20	10	---------	---------	MT only Control
D1	27.5	---------	2.5	---------	KHC only Control
E1	27.5	---------	2.5	---------	KHC only Control
F1	17.5	10	2.5	---------	KHC ATPase #1
G1	17.5	10	2.5	---------	KHC ATPase #2
H1	17.5	10	2.5	---------	KHC ATPase #3
A2	To 30 µl final volume	---------	---------	0.2 µg	Kinesin Control #1
B2	To 30 µl final volume	---------	---------	0.4 µg	Kinesin Control #2
C2	To 30 µl final volume	---------	---------	0.6 µg	Kinesin Control #3
D2	To 30 µl final volume	---------	---------	0.8 µg	Kinesin Control #4
E2	To 30 µl final volume	---------	---------	1.0 µg	Kinesin Control #5
F2	To 30 µl final volume	10	---------	0.2 µg	Kinesin test #1A
G2	To 30 µl final volume	10	---------	0.2 µg	Kinesin test #1B
H2	To 30 µl final volume	10	---------	0.4 µg	Kinesin test #2A
A3	To 30 µl final volume	10	---------	0.4 µg	Kinesin test #2B
B3	To 30 µl final volume	10	---------	0.6 µg	Kinesin test #3A
C3	To 30 µl final volume	10	---------	0.6 µg	Kinesin test #3B
D3	To 30 µl final volume	10	---------	0.8 µg	Kinesin test #4A
E3	To 30 µl final volume	10	---------	0.8 µg	Kinesin test #4B
F3	To 30 µl final volume	10	---------	1.0 µg	Kinesin test #5A
G3	To 30 µl final volume	10	---------	1.0 µg	Kinesin test #5B
H3	To 30 µl final volume	10	---------	1.0 µg	Kinesin test #5C

** The microgram amount of any given kinesin will need to be determined by protein titration. It is recommended to begin by titrating you protein between 0.2 µg and 1 µg per assay.

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. When carrying out low numbers of reactions, such as the 24 well reactions shown above, the following procedure is recommended:

a) **BEFORE ADDING THE ATP** make sure that the spectrophotometer is set up correctly in end point read mode at 650 nm wavelength.
b) Immediately prior to use, aliquot 100 µl of the diluted ATP solution into 8 empty wells of a 96 well plate (for example wells A12 - H12).
c) Use an 8 well multichannel pipettor to aliquot 5 µl of ATP per reaction (0.3 mM final).
d) Allow the reactions to proceed at room temperature for exactly 5 minutes.

Terminating and Reading the Reactions

1) Terminate the reactions by adding 70 µl of room temperature CytoPhos to each well.
2) Allow this to react for 10 minutes and take readings at 650 nm. Designate the reaction in well A1 (Table 2) 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.

Control Reactions

The MT only and kinesin only reactions should give very low readings that are at least 10 fold lower than the kinesin ATPase reactions. These reactions will often generate a slightly negative reading.

Kinesin ATPase Reactions

Kinesin ATPase rates are often expressed as nmoles of ATP hydrolyzed per minute per mg of kinesin protein. Under the conditions described in Section VI, the control kinesin heavy chain motor domain protein should give a rate of 1200 – 1500 nmoles/min/mg.

VII: References

1) Goldstein, L. Annu. Rev. Genet. 27: 319-351 (1993)
2) Sawin, K.E. & Scholey, J.M. Trends in Cell Biol. 1: 122-129 (1991)
3) Kuznetsov, S.A., & Gelfand, V.I. Proc. Natl. Acad. Sci. USA 83: 8530-8534 (1986)
4) Kodama, T. et al. J. Biochem. 99: 1465-1472 (1986)

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