Kinesin ELIPA Biochem Kit

Cat. # BK060

Phone: (303) 322.2254 **Fax:** (303) 322.2257
Customer Service: cserve@cytoskeleton.com
Technical Support: tservice@cytoskeleton.com

Manual Contents

I: Kinesin ELIPA 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 ELIPA (Enzyme Linked Inorganic Phosphate Assay) Biochem Kit. The assay is an adaptation of a method originally described by Webb for the measurement of glycerol kinase plus D-glyceraldehyde ATPase activity and for actin activated myosin ATPase (4). The assay is based upon an absorbance shift (330 - 360 nm) that occurs when 2-amino-6-mercapto-7-methylpurine ribonucleoside (MESG) is catalytically converted to 2-amino-6-mercapto-7-methyl purine in the presence of inorganic phosphate (Pi). The reaction is catalysed by purine nucleoside phosphorylase (PNP). One molecule of inorganic phosphate will yield one molecule of 2-amino-6-mercapto-7-methyl purine in an essentially irreversible reaction (5). Thus, the absorbance at 360 nm is directly proportional to the amount of Pi generated in the kinesin ATPase reaction. **Figure 1** shows a typical set of results from a kinesin heavy chain motor domain protein MT activated ATPase ELIPA.

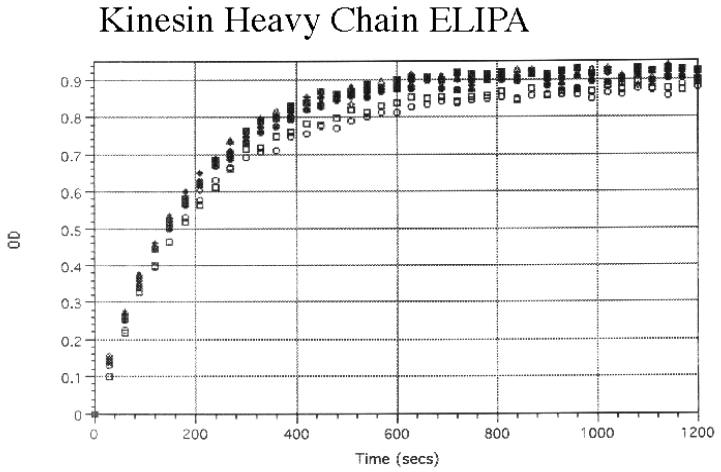


Figure 1. Kinesin Heavy Chain ELIPA. *Method: The reactions were conducted in a 96 well plate format (300 µl reaction volumes). Each reaction contains 94 nM kinesin heavy chain motor domain protein (cat # KR01), 0.66 µM taxol stabilized microtubules (cat # MT002), 0.2 mM MESG, 0.3 U PNP, 15 µM taxol, 15 mM PIPES pH 7, 5 mM MgCl2, 0.6 mM ATP. Control reactions were carried out in the absence of motor protein and in the absence of MTs, these reactions gave readings of <0.02 (data not shown). Reactions were measured in a SpectraMax 250 (Molecular Devices) set in kinetic mode and 360 nm absorbance wavelength. Readings were taken at room temperature once every 30 seconds for a total reaction time of 20 minutes.*

II: Important Technical Notes

Notes on Updated Manual Version

v4.0: Updated to new format version on 8/11/2021.

V5.0: Phosphate standard (0.5 mM) was converted from a liquid to a lyophilized formulation to prolong shelf life. The standard PI05-L is resuspended in 1 ml of deionized water to give a 0.5 mM phosphate solution (see instructions in Section IV).

II: Important Technical Notes

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

ELIPA Reagents

1) This kit contains sufficient ELIPA reagents to carry out approximately 100 reactions (300 µl volume). This corresponds to a complete microtiter plate of 300 µl well volume. More reactions can be achieved if half area wells are used. The kit contains excess of the ELIPA reagents that are required to perform the standard Pi curve.

2) The kinesin heavy chain motor domain control protein contained in the kit provides sufficient reagent for 12 control assays. The remaining assays will require motor proteins that are provided by the end user. A wide selection of kinesin motor proteins are available from Cytoskeleton Inc. and can be purchased separately (see Section VIII).

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

ELIPA Optimization

The Kinesin ELIPA kit has been developed to provide a good general substrate for a broad range of kinesin motor domain proteins. For example, using this kit as outlined in the ELIPA Protocol (Section V) will result in a Vmax value of 7,000 - 8,000 nmoles of ATPs hydrolysed per minute per mg of kinesin heavy chain motor domain protein. The same assay, using 235 nM of Eg5 motor domain protein results in a Vmax value of 1000 nmoles of ATPs hydrolysed per minute per mg of Eg5 motor domain protein. These values are comparable to published data (6). It should be noted, however, that optimization of the ELIPA assay may be needed for any given motor protein or motor protein construct. It is recommended to begin optimization by titrating the amount of motor protein and ATP in the assay.

Instrumentation

The assay is based upon an absorption shift from 340 nm to 360 nm. It is therefore very important to use a spectrophotometer that has a narrow bandwidth in order that the wavelength for reading the assay does not encroach upon the 340 nm range. It is recommended that a monochromatic spectrophotometer such as a SpectroMax 350 (Molecular Devices) be used when possible as the bandwidth in these machines is very narrow (2-5 nm). If a filter based system is being used then it is important to make sure that the filter bandwidth is less than 10 nm.

III: Kit Contents

KIT COMPONENT	DESCRIPTION
ELIPA Reaction Buffer	One bottle containing 30 ml of buffer. Composition, 15 mM PIPES pH 7, 5 mM $MgCl_2$.
ELIPA Reagent 1	One bottle, lyophilized. Contains 20 µmoles of 2-amino-6-mercapto-7-methylpurine riboside (MESG).
ELIPA Reagent 2	One tube. Contains 50 units of purine nucleoside phosphorylase (PNP). One unit of PNP will cause the phosphorolysis of 1 µmole of inosine to hypoxanthine and ribose 1-phosphate per minute at pH 7.4 at 25°C.
Phosphate standard (Part # PI05-L)	One tube-lyophilized. When reconstituted will give 1 ml of 0.5 mM phosphate standard (KH_2PO_4).
ELIPA Reagent 1 Resuspension Buffer	One bottle.
Kinesin Heavy Chain Motor Domain protein (Cat # KR01-A)	One tube, 25 µg of lyophilized protein. Used as positive control for the ELIPA assay. Sufficient reagent for 12 positive control assays.
Microtubules (Cat # MT002)	Four tubes, each contains 500 µg of lyophilized microtubules (MT's)
Taxol (Cat # TXD01)	Four tubes, lyophilized. When reconstituted each tube contains 100 µl of 2 mM Taxol.
Anhydrous DMSO	One tube, 2 ml. Used to resuspend the Taxol.

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
ELIPA Reaction buffer	Not required.	Store at 4°C. Stable for six months under these conditions.
ELIPA Reagent 1	1) Warm the 20 ml of ELIPA Reagent 1 Resuspension Buffer to room temperature and reconstitute the ELIPA Reagent 1. 2) This component does not resuspend easily and should be shaken every 1-2 minutes. **DO NOT** heat to resuspend. After 10 minutes the reagent can be aliquoted even if a little reagent is left that will not resuspend. 3) Aliquot into 40 x 500 µl volumes. 4) Freeze immediately by placing at -20° C.	Store at -20°C. Stable for at least one month under these conditions.
ELIPA Reagent 2	Reconstitute with 500 µL of sterile distilled water.	Store at 4°C. Stable for at least one month under these conditions.
Phosphate standard	Resuspend in 1 ml of phosphate free deionized water to give a 0.5 mM phosphate standard.	Store at 4°C. Stable for at least one year under these conditions.
ELIPA Reagent Resuspension Buffer	Not required	Store at 4°C. Stable for at least one year under these conditions.
ATP Stock	1) ATP stock is not provided with this kit. Cytoskeleton ATP (catalog # BSA04) is **NOT suitable** for this assay. We recommend Sigma ATP (Cat.# A3377). 2) Make up a stock of ATP to 100 mM at pH 7.0. 3) Aliquot into 83 µl sized volumes and freeze at -20°C. 4) It is important not to freeze / thaw aliquots of ATP as hydrolysis to ADP may inhibit kinesin ATPase activity.	Store at -20°C. Stable for at least 1 year under these conditions.
Kinesin Heavy Chain Motor Domain protein (Cat # KR01-A)	1) Reconstitute in 10 µl of ELIPA Reaction Buffer to give a 2.5 mg/ml protein concentration. 2) Aliquot into 2 x 5 µl sizes 3) Snap freeze in liquid nitrogen.	

IV: Things to do Prior to Beginning the Assay

Continued from the previous page:

KIT COMPONENT	RECONSTITUTION	STORAGE CONDITIONS
Microtubules (Cat # MT002)	1) Warm 2 ml of ELIPA Reaction Buffer to room temperature by placing in a 37°C water bath for 20 minutes. 2) Supplement with 20µl of Taxol stock solution and mix well. 3) Reconstitute each tube of Microtubules with 500 µl of the Taxol supplemented buffer. 4) Each tube of MT's is sufficient for 3 lanes of a 96 well plate (24 wells). 5) The reconstituted MT's can be aliquoted into 160 µl sizes and snap frozen in liquid nitrogen. 6) Alternatively, the reconstituted MT's are stable for 2-3 days at room temperature so each tube can be reconstituted as needed in 500 µl of Taxol supplemented ELIPA Reaction Buffer.	Store frozen MT's at -70° C. Stable for at least 6 months under these conditions. Store reconstituted MT's at room temperature. Stable for 2-3 days under these conditions.
Taxol (Cat # TXD01)	Reconstitute each tube with 100 µl of anhydrous DMSO (supplied in kit). This solution will freeze when placed on ice so use at room temperature.	Store at -20°C. Stable for at least 1 year under these conditions.
Anhydrous DMSO	Not required.	Store at room temperature. Stable for atleast 1 year under these conditions.

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 2 nmoles to 50 nmoles of Pi. Each µl of the supplied phosphate standard is equivalent to 0.5 nmoles of Pi.

Method

1) Add the reagents shown in **Table 1** to the wells of a 96 well plate and incubate for 15 minutes at room temperature.

2) Set the spectrophotometer to read an end-point assay at absorbance 360 nm.

   NOTE: The reaction is based upon a shift in absorbance from 340 nm to 360 nm. Your spectrophotometer should therefore be set at an absorbance wavelength of 360 nm for readings. If using a filter based machine, check that the bandwidth of the filter is no more than 10 nm or you may experience significant background noise and greatly reduced sensitivity of the assay.

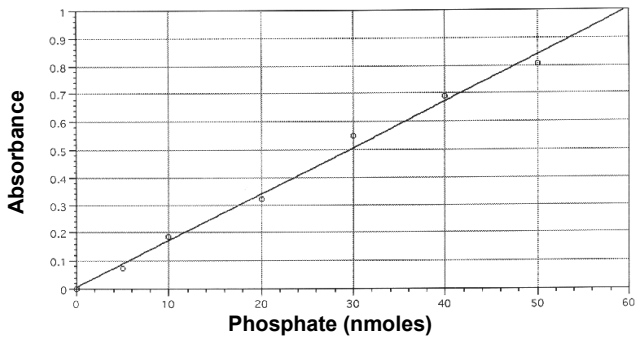
3) Designate well A1 as the BLANK and read the plate.

4) Plot a Phosphate Standard curve from your results (see **Figure 2**).

Table 1: Standard Pi Curve Reactions

Well Allocation	ELIPA Reaction Buffer (µl)	ELIPA Reagent 1 (µl)	ELIPA Reagent 2 (µl)	0.5 mM Phosphate Standard (µl)	n moles of Pi per well
A1	237	60	3	zero	BLANK
B1	237	60	3	zero	0
C1	227	60	3	10	5
D1	217	60	3	20	10
E1	197	60	3	40	20
F1	177	60	3	60	30
G1	157	60	3	80	40
H1	137	60	3	100	50

Figure 2: Standard Pi Curve Generated From The Above Reactions



VI: ELIPA Protocol

Protocol for one lane of a 96 well plate (8 wells total). The ELIPA reaction is first set up with all of the reaction components, minus ATP. The reaction is started by the addition of ATP. If you wish to perform more than 8 reactions, simply scale up the reaction conditions shown below;

Instrumentation Settings and Microtiter Plates

The reaction is based upon a shift in absorbance from 340 nm to 360 nm. Your spectrophotometer should therefore be set at an absorbance wavelength of 360 nm for readings. If using a filter based machine, check that the bandwidth of the filter is no more than 10 nm or you may experience significant background noise and greatly reduced sensitivity of the assay. The spectrophotometer should be at room temperature and set on kinetic mode, it is recommended to take a reading once every 30 seconds. There is no need to elect a blank well as the reaction minus motor will serve as a background control (see next section). Do not pre-read the microtiter plate. Start the kinetic readings at time zero. Under the experimental conditions described in this manual, 1 µg of kinesin heavy chain motor domain protein will produce an absorbance maximum of approximately 0.8 OD_{360} units in 15 minutes.

As this assay measures inorganic phosphate (Pi) generation, one needs to make sure that clean Pi free plates are used.

Reaction Setup

1) Aliquot 2 ml of ELIPA Reaction Buffer from 4°C storage and warm to room temperature by placing in a 37°C water bath for 10 minutes.

2) Thaw out one 500 µl aliquot of ELIPA reagent 1 by placing in a room temperature water bath for 5 minutes. Place on ice.

3) Thaw out one 83 µl aliquot of 100 mM ATP stock, dilute with 750 µl of ice cold ELIPA Reaction Buffer. Place on ice. **NOTE: CYTOSKELETON ATP (Cat. # BSA04) IS NOT SUITABLE IN THIS ASSAY.**

4) Thaw out one 160 µl aliquot of MT's by placing them in a room temperature water bath for 10-15 minutes. Alternatively, resuspend one lyophilized tube of MT's in 500 µl of Reaction Buffer (room temperature) plus 5 µl of Taxol stock. Store these at room temperature. The MT's will be stable for 1-2 days.

5) Thaw out one aliquot of taxol stock and store at room temperature.

6) Remove the ELIPA Reagent 2 from 4°C and place on ice.

7) Thaw out one 5 µl aliquot of Kinesin protein and dilute this with 45 µl of ice cold ELIPA reaction Buffer. Place on ice.

8) Mix the components together **IN THE ORDER SHOWN BELOW**:

MT ELIPA MIX:	
ELIPA Reaction Buffer (room temperature)	2 ml
Taxol Stock	20 µl
MT's	160 µl
ELIPA Reagent 1	480 µl
ELIPA Reagent 2	24 µl

9) Agitate the MT ELIPA MIX for 5 minutes on a room temperature rotator or rocker.

10) The MT ELIPA MIX is now ready for the addition of your motor protein. The example reactions given in **Table 2** use the kinesin control protein (Cat # KR01) in all of the motor ATPase experimental lanes.

Table 2: Kinesin ELIPA Assay using Kinesin Heavy Chain Control Protein (Cat # KR01)

Well Designation	MT ELIPA MIX (µl)	MOTOR PROTEIN (µl)
A1	300	zero control well
B1	300	4 µl KR01 (1 µg) **
C1	300	4 µl KR01 (1 µg)
D1	300	4 µl KR01 (1 µg)
E1	300	4 µl KR01 (1 µg)
F1	300	4 µl KR01 (1 µg)
G1	300	4 µl KR01 (1 µg)
H1	300	4 µl KR01 (1 µg)

*** If your reaction is using the same motor in all experimental wells, the motor (28 µl total) can be pre-mixed with the MT ELIPA MIX prior to aliquoting into the reaction wells. This method of motor addition improves assay reproducibility.*

Starting the Reaction

The MT activated kinesin 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 8 well reaction format shown above, the following procedure is recommended;

a) **BEFORE ADDING THE ATP** make sure that the spectrophotometer is set up correctly in kinetic read mode at 360 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 20 µl of ATP per ELIPA reaction.

d) Immediately read the reaction on a kinetic setting at 360 nm wavelength.

Interpretation of Experimental Results

The ATP'ase rate can be determined by calculating the amount of Pi generated using the standard Pi curve described in **Section V**. The ATPase rate is often expressed as nmoles of ATP hydrolysed per minute per mg of motor protein.

VII: References

1) Vale, R.D. & Fletterick, R.J. 1997. The Design Plan of Kinesin Motors. Ann. Rev. Cell Dev. Biol. 13: 745-777.

2) Endow, S. A. 1999. Microtubule Motors in Spindle and Chromosome Motility. Eur. J. Biochem 262: 12-18.

3) Johnson, K.A. & Gilbert, S.P. 1995. Pathway of the Microtubule-Kinesin ATPase. Biophysical Journal 68: 173s - 179s.

4) Webb, M.R. 1992. A continuous spectrophotometric assay for inorganic phosphate and for measuring phosphate release kinetics in biological systems. Proc. Natl. Acad. Sci. USA 89: 4884-4887.

5) Analytical Biochemistry 1997 246: 86-95

6) Lockhart, A & Cross, R.A. 1996. Kinetics and Motility of the Eg5 Microtubule Motor. Biochemistry 35: 2365 - 2373.

VIII: Related Motor Werks Products

Cytoskeleton Motor Werks (CMW) reagents are a set of tools developed to specifically aid researchers in the study of Molecular Motors.

ITEM CAT #	MOTOR PROTEIN	KINESIN CLASS	QUANTITY
KR01	HUMAN HEAVY CHAIN	N-I	2 x 25 ug 10 x 25 ug 1 x 1 mg
EG01	EG5	N-II	2 x 25 ug 10 x 25 ug 1 x 1 mg
BM01 **NOT HUMAN**	BimC *Aspergillus nidulans*	N-II	2 x 25 ug 10 x 25 ug 1 x 1 mg
CP01	CENP-E	N-VII	2 x 25 ug 10 x 25 ug 1 x 1 mg
CR01	Chromokinesin	N-V	2 x 25 ug 10 x 25 ug 1 x 1 mg
KC01	KIFC3	C-II	2 x 25 ug 10 x 25 ug 1 x 1 mg
KF01	KIF3C	N-IV	2 x 25 ug 10 x 25 ug 1 x 1 mg
MK01	MCAK	M	2 x 25 ug 10 x 25 ug 1 x 1 mg
MP01	MKLP 1	N-VI	2 x 25 ug 10 x 25 ug 1 x 1 mg

Limited Use Statement

The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of product and components of product in research conducted by the buyer. The buyer cannot sell or otherwise transfer this product or any component thereof to a third party or otherwise use this product or its components for commercial purposes. Commercial purposes include, but are not limited to: use of the product or its components in manufacturing; use of the product or its components to provide a service; resale of the product or its components.

The terms of this Limited Use Statement apply to all buyers including academic and for-profit entities. If the purchaser is not willing to accept the conditions of this Limited Use Statement, Cytoskeleton Inc. is willing to accept return of the unused product with a full refund.

Phone: (303) 322.2254 **Fax:** (303) 322.2257
Customer Service: cserve@cytoskeleton.com
Technical Support: tservice@cytoskeleton.com