V. 4.0

ATPase/GTPase ELIPA Biochem Kit

Cat. # BK051/BK052

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

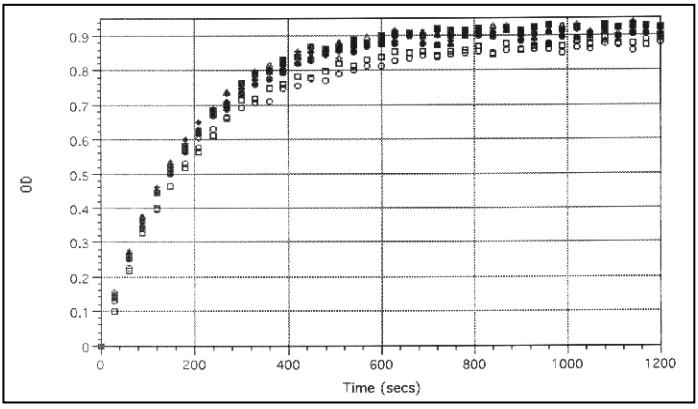
Manual Contents

I: ATPase/GTPase ELIPA Introduction

There are a multitude of enzymes that hydrolyze ATP or GTP to form ADP or GDP and inorganic phosphate (Pi). The Enzyme Linked Inorganic Phosphate Assay (ELIPA) from Cytoskeleton Inc. allows one to measure the phosphate released during hydrolysis on a real time basis. Thus a kinetic assay technique is produced for your enzyme's activity. Particular purified enzymes that are applicable to this analysis are signaling phosphatases, apyrase, kinesin motors (see Fig. 1), metabolic enzymes, membrane transporters and alkaline phosphatase. Generally this assay is useful for enzymes with Kcat above 0.1, if your enzyme or the family of enzymes has a lower Kcat then the CytoPhosTM Phosphate Assay (Cat. # BK054) is useful.

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 (1). 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 catalyzed 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 (2). Thus, the absorbance at 360 nm is directly proportional to the amount of Pi generated in the reaction. Figure 1 shows a typical set of results from a kinesin heavy chain motor domain protein (Cat. KR01), the ATPase activity is catalyzed by the addition of microtubules (Cat. # MT002).

Figure 1: Kinesin Heavy Chain ELIPA Assay



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 7.0, 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 at 360 nm absorbance wavelength. Readings were taken at room temperature once every 30 s for a total reaction time of 20 min.

II: Purchaser Notification

Limited Use Statement

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III. 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 (Corning Costar # 3696 or 3697). The kit contains excess of the ELIPA reagents that are required to perform the Pi standard curve.
2) A phosphate standard is included in the kit for producing a standard curve (see Section V).
3) Some 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 ELIPA Reagent 1) is not recommended as it will result in inactivation of the reagents.

ELIPA Optimization

The ELIPA kit has been developed to provide a good general substrate for a broad range of ATPases with Kcat of >0.1. Kcat is a measure of specific activity at the molecular level, a Kcat of 1.0 means that one molecule of ATPase protein will hydrolyze one molecule of ATP per second. This measure is usually a maximum possible activity under optimal conditions, it is more reasonable to expect slower (possibly 50% activity) reaction rates for comparative assays. It should be noted that optimization of the assay may be needed for any given ATPase. An optimization procedure is given in Appendix #1.

Equipment Required

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 SpectraMax 250 (Molecular Devices Inc.) 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 is 370 nm with bandwidth is less than 10 nm.

Materials Required

1. Test protein at 1 mg/ml and >90% purity, or lower purity with increased total protein concentration to compensate for the lower purity. The protein must be in a phosphate free buffer such as Tris-HCl (PBS is not suitable).
2. Protein cofactor or buffer condition. (e.g. microtubules for motor proteins, sometimes an alkaline pH buffer, or a certain ion such as calcium for FtsZ). This reagent must also be free of phosphate ions.

IV: Kit Contents

This kit contains sufficient reagents for approximately 100 assays of 300 µl volume. The kit arrives at room temperature and can be stored desiccated at 4°C.

Kit Component	**Cat.# or Part#***	**Quantity**	**Storage Upon Arrival**
ELIPA Reaction Buffer	Part# EREC	1 bottle, 30 ml. Composition, 15 mM PIPES pH 7.0, 5 mM MgCl2	4°C
ELIPA Reagent 1	Part# ELP01	1 bottle, lyophilized. Contains 20 µmoles of 2-amino-6-mercapto-7-methylpurine riboside (MESG).	Desiccated 4°C
ELIPA Reagent 2; Purine Nucleotide Phosphorylase	Part# ELP03	1 tube, lyophilized.	Desiccated 4°C
0.5mM Phosphate Standard	Part # PI05	1 tube, 1 ml. 0.5 mM phosphate standard (KH_2PO_4).	4°C
ELIPA Reagent 1 Re-suspension Buffer	Part # ERSP	1 bottle, 20 ml. 3.0 mM acetic acid.	4°C
ATP Stock	Cat. # BSA04	1 tube, lyophilized. When reconstituted; 1 ml of 100 mM ATP.	Desiccated 4°C
GTP Stock	Cat # BST06	2 tube, lyophilized. When reconstituted; 100 µl of 100 mM GTP per tube	Desiccated 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.

V: Things to do Prior to Beginning the Assay

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

Reagents	**Reconstitution**	**Storage**
ELIPA Reaction Buffer	None required.	Store at 4°C. Stable for at least 6 months under these conditions.
Phosphate Standard	None required.	Store at 4°C. Stable for at least 12 months under these conditions.
ELIPA Reagent 1	1) Reconstitute the ELIPA Reagent 1 with 20 ml of ELIPA Reagent 1 Resuspension Buffer. 2) This component does not resuspend easily and should be shaken every 1-2 min. **DO NOT** heat to resuspend. After 10 min the reagent can be aliquoted even if a little reagent is left that will not resuspend. 3) Aliquot into 20 x 1 ml volumes. 4) Freeze immediately by placing at -70° C.	Store reconstituted reagent at -70°C. Stable for six months under these conditions. Dry reagent can be stored desiccated at 4°C.
ELIPA Reagent 2	1) Reconstitute with 500 µl of sterile distilled water.	Store reconstituted reagent at 4°C. Stable for one month under these conditions. Dry reagent can be stored desiccated at 4°C.
ATP Stock	1) Reconstitute in 1 ml of ice cold 100 mM Tris pH 7.5. This stock will take up to 1 min to completely resuspend so keep on ice for 1 minute with occasional pipetting to help resuspension. 2) Aliquot into 20 x 50 µl volumes. 3) Snap freeze in liquid nitrogen and store at -70°C.	Store reconstituted reagent at -20°C. Stable for six months under these conditions. Dry reagent can be stored desiccated at 4°C.
GTP Stock	1) Reconstitute each tube in 100 µl of ice cold sterile distilled water. This stock will take up to 1 minute to completely resuspend so keep on ice for 1 min with occasional pipetting to help resuspension. 2) Aliquot each tube into 5 x 20 µl volumes. 3) Snap freeze in liquid nitrogen and store at -70°C.	Store at -20°C. Stable for six months under these conditions. Dry reagent can be stored desiccated at 4°C.

VI: 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 2 nmoles to 50 nmoles of Pi. Each microliter of the 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 min 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. The spectrophotometer should therefore be set at an absorbance wavelength of 360 nm for readings. If using a filter based machine, you need a filter of 370 nm and a bandwidth of the filter of no more than 10 nm, if these conditions are not met you may experience significant background noise and greatly reduced sensitivity of the assay.

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

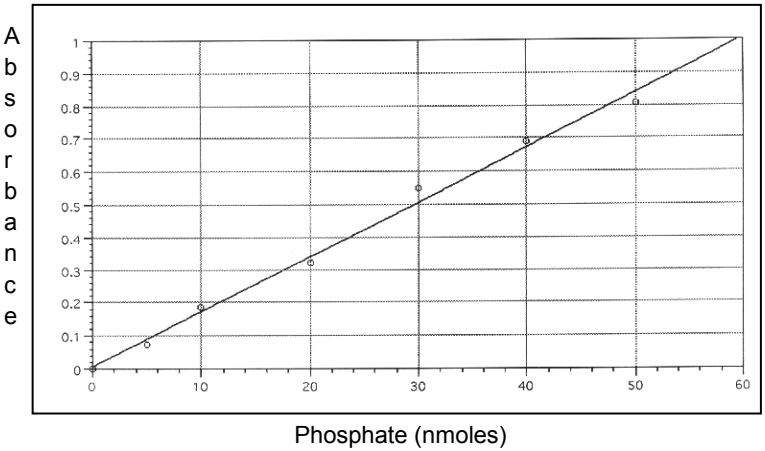
4) Subtract the BLANK readings from all other data, then plot a phosphate standard curve from the results (see Figure 2).

Table I: Phosphate Standard Curve Reactions

Well Allocation	Reaction Buffer (µl)	ELIPA Reagent 1 (µl)	Dilute ELIPA Reagent 2 (µl)	0.5 mM Phosphate Standard (µl)	nmoles of Pi per well
A1	237	60	3	0	0
B1	237	60	3	0	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

VI: Phosphate Standard Curve (continued)

Figure 2: Phosphate Standard Curve Generated From The Above Reactions



Method 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, then the filter must be 370 nm with a bandwidth of 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 or 37ºC depending on your enzyme and set on kinetic mode, it is recommended to take a reading once every 30 s. There is no need to elect a blank well as the reaction minus enzyme will serve as a background control (see next section). Do not pre-read the microtiter plate. Start the kinetic readings at time zero. Make sure that all equipment and pipettes are clean and free of Pi, as this assay measures inorganic phosphate (Pi) generation.

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 min.
2) Thaw one 1 ml aliquot of ELIPA Reagent 1 by placing in a beaker of water for 5 min, then place on ice.
3) Thaw one aliquot of ATP or GTP stock, dilute with 450 µl (ATP) or 180 µl (GTP) of ice cold Reaction Buffer, then place on ice. This gives a concentration of 10 mM for each nucleotide solution.
4) Remove the ELIPA Reagent 2 from 4°C and defrost in a beaker of water for 1 min, then place on ice.
5) Prepare your protein of interest and place on ice.
6) Add the following components in the order they are shown:

ELIPA MIX

ELIPA Reagent 2	25 µl
ELIPA Reaction Buffer (room temperature)	2 ml
ELIPA Reagent 1	480 µl

Mix by inverting three time and place at room temperature on the bench.

7) The ELIPA MIX is now ready for the addition of your test protein*. It is recommended to titrate the protein amounts for the initial activity studies; an example of a standard protein titration is given in Table 2. Remember to keep the volumes equal between wells by making the volume up with a protein compatible buffer. This buffer cannot contain phosphate because that would interfere with the ELIPA detection mechanism.

** If your reaction requires another cofactor or protein, then this component should be added here. For example, molecular motors require microtubules, or alkaline phosphatase requires alkali pH so you would add a final concentration of 100 mM of $NaHCO_3$ buffer at pH 9.0 (i.e. 30 µl of a 1 M stock).*

Table 2: Example protein titrations for optimization of ATPase/GTPase activity:

WELL	MT ELIPA MIX (µl)	TEST PROTEIN	TEST BUFFER (µl)
A1	270	0 µl, control well	30
B1	270	10 µl protein at 0.001 mg/ml	20
C1	270	30 µl protein at 0.001 mg/ml	0
D1	270	10 µl protein at 0.01 mg/ml	20
E1	270	30 µl protein at 0.01 mg/ml	0
F1	270	10 µl protein at 0.1 mg/ml	20
G1	270	30 µl protein at 0.1 mg/ml	0
H1	270	10 µl protein at 1.0 mg/ml	20

Note: If your reaction requires another cofactor or protein, then this component should be added here. For example, molecular motors require microtubules, or alkaline phosphatase requires alkali pH so you would add a final concentration of 100 mM of $NaHCO_3$ buffer at pH 9.0 (i.e. 30 µl of a 1 M stock).

Starting the Reaction

The ELIPA reaction is started by the addition of ATP or GTP. It is therefore highly advisable to add ATP or GTP using a multichannel pipettor. In this way all reaction will begin simultaneously. When carrying out low numbers of reactions, such as the 8 well reactions 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 25 µl of the diluted ATP or GTP solution (10 mM) into 8 empty V-shaped wells of a 96 titration plate.
C) Use an 8 well multichannel pipettor to aliquot 20 µl of ATP or GTP per ELIPA reaction. This gives a final nucleotide concentration of 0.7 mM. NOTE: it is also recommended to titrate the ATP or GTP to optimize enzyme hydrolysis.
D) Immediately read the reaction on a kinetic format, using 40 readings with intervals of 30 s each.

Interpretation of Experimental Results

The ATPase rate can be determined by calculating the amount of Pi generated using the phosphate standard curve described in section VI. The ATPase/GTPase rate is often expressed as nmoles of ATP / GTP hydrolyzed per min per mg of protein.

VIII: References

1) 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.

2) Cheng Q., Wang Z-X., and Killilea SD. 1997. A continuous spectrophotometric assay for protein phosphatases. Analytical Biochemistry **226**: 68-73.

IX: Related Phosphate Assays

HTS Kinesin ATPase Endpoint Assay	Cat. # BK053	KIT 1000 assays
CytoPhos™ Phosphate Assay (1-500 µg/ml protein reactions)	Cat. # BK054	KIT 1000 assays
Kinesin ELIPA (Enzyme Linked Inorganic Phosphate Assay)	Cat. # BK060	KIT 96 assays

Appendix: Assay Optimization

There are several parameters that may need to be optimized for any given ATPase/ GTPase reaction; the most important parameters for consideration are listed below:

1) **Protein concentration**: A titration of protein amount per assay is highly recommended. A good range is between 0.1 µg – 20 µg per assay.

2) **Nucleotide concentration**: The standard ATPase/GTPase assay described in this manual uses approximately 0.7 mM final concentration of ATP or GTP per assay. It is recommended to titrate the nucleotide concentration. A good range is between 0.3 – 3 mM final per assay.

3) **Reaction Buffer**: The ELIPA reaction buffer supplied in this kit has the following composition: 15 mM PIPES buffer pH 7.0, 5 mM $MgCl_2$. This buffer may not be compatible with the ATPase/GTPase of interest. Reaction buffer can be changed to suit the enzyme of interest, however, phosphate containing buffers MUST BE AVOIDED as they will interfere with the ELIPA assay. In this regard it is also important to make up proteins and buffers in phosphate free water. Milli Q water is suitable for reagent preparation.

4) Important buffer parameters that should be optimized include salt concentration, pH, co-factor requirements e.g. Mg^{2+} ions. All of these parameters should be titrated for optimal results.

NOTES:

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