PROTOCOL

High Throughput Colorimetric ATPase Assay Kit

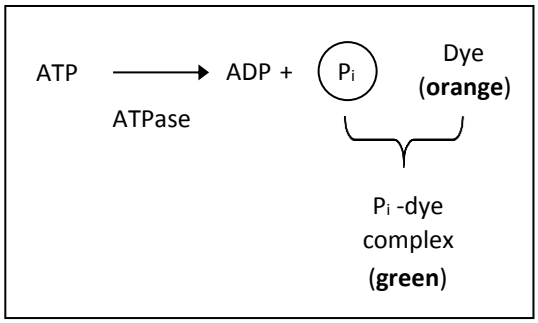
Applicable to: 601-0120 2 plates
601-0121 5 plates

Release 1 © EXPEDEON 28/02/2018

INTRODUCTION

The ATPase colorimetric assay kit employs a 96-well plate format with all of the reagents necessary for measuring ATPase activity. The kit also contains PiColorLock™ (a superior malachite green reagent) which has special additives to prevent backgrounds arising from non-enzymatic ATP hydrolysis.

Fig 1. Principle of the ATPase assay **kit**



KIT CONTENTS AND STORAGE

Components in the 2-plate ATPase assay kit (5-plate kit amounts in brackets)

Store at 4°C:

- 1 x 10ml of PiColorLock™ * (1 x 25ml)
- 1 x 0.25ml of Accelerator (1 x 0.5ml)
- 1 x 5ml of Stabilizer (1 x 10ml)
- 1 x 1.5ml of 0.1M MgCl2 (2 x 1.5ml)
- 1 x 5ml of 0.5M Tris pH 7.5 (1 x 10ml)
- 1 x 5ml of 0.1mM Pi standard (1 x 10ml)

Note*: Take care, the solution is very acidic

Store at -20 or -70°C:

4 vials of lyophilized ATP (10 vials)

Store at room temperature:

2 x 96-well plates (5 plates)

INSTRUCTIONS

Overview of ATPase colorimetric assay

(a) Make substrate/buffer mix.

(b) Make PiColorLock™ mix by adding Accelerator to PiColorLock™ reagent just before use.

(c) Set up assays with your ATPase; add 100µl enzyme to 100µl substrate/buffer mix.

(d) Add 50µl of PiColorLock™ mix to stop reactions.

(e) After 2 minutes, add 20µl of Stabilizer and mix thoroughly by pipetting up and down.

(f) After 30 minutes, read the plate at a wavelength in the range 590-660nm.

1. Preparation of reagents

1.1 ATP substrate

Reconstitute the lyophilized ATP to 10mM concentration by adding the volume of MQ H_2O stated on the vial label. Note: **This reconstitution volume is batch specific.** Keep the ATP on ice when not in use and snap freeze any surplus reagent (aliquot to avoid multiple freeze-thaw cycles) and store at -70°C.

1.2 Substrate/buffer (SB) mix

The assay kit is supplied with 0.5M Tris assay buffer pH 7.5, but you can substitute any other non-phosphate-containing buffer to suit your particular ATPase. All ATPases will require a metal ion cofactor, often Mg^{2+} (0.1M $MgCl_2$ is supplied with the kit) but again you can substitute your own metal ion, or include other metal ions, as required.

Table 1 (below) shows the volumes required to make up SB mix for selected numbers of wells. The ATP concentration in this SB mix is 1mM. Once the enzyme has been added to the SB mix, the final buffer, Mg^{2+} and ATP concentrations are 50mM, 2.5mM, and 0.5mM, respectively.

Table 1. Preparation of SB mix

No. of Wells	0.5M Buffer (µl)	0.1M $MgCl_2$ (µl)	10mM ATP (µl)	MQ Water (µl)
1	20	5	10	65
25	500	125	250	1625
50	1000	250	500	3250
75	1500	375	750	4875
100	2000	500	1000	6500
150	3000	750	1500	9750
200	4000	1000	2000	13000
250	5000	1250	2500	16250
500	10000	2500	5000	32500

1.3 PiColorLock™ mix

Prepare the mix immediately before the reagent is required by adding 1/100 vol. of Accelerator to PiColorLock™. Table 2 (below) gives the volumes of reagent required for the specified numbers of wells. The PiColorLock™ mix *cannot* be stored for long periods; make up only what you will use on the day.

Table 2. Volumes needed to make PiColorLock™ mix

No. of Wells	PiColorLock™ (µl)	PiColorLock™ (µl)
1	50	0.5
25	1250	12.5
50	2500	25
75	3750	37.5
100	5000	50
150	7500	75
200	10000	100
250	12500	125
500	25000	250

2. Assay procedure

The procedure involves mixing 100µl of SB mix with 100µl of appropriately diluted enzyme and incubating for a fixed period of time at a set temperature. The reaction is then stopped by adding 50µl of P_iColorLock™ mix. After 2 minutes, the stabilizer is added and mixed in thoroughly by pipetting up and down. A green colour develops for samples with P_i. The colour reaches a maximum signal at 30 minutes and the plate can then be read at any wavelength from 590-650nm.

2.1 How to determine assay conditions

There are no strict guidelines and the key parameters (i.e. enzyme dilution, assay time and temperature) will depend on the intrinsic activity of the particular enzyme, the concentration of the enzyme, and operator preferences with regard to assay time and temperature. The enzyme sample and ATP are usually incubated for between 15 min and 1 hour before addition of the P_i-detection reagent; very short assay times with very concentrated enzyme should be avoided. Plate assays are more easily performed at room temperature than at 37°C. It is important to operate in the 'linear range' of the assay i.e. under conditions where doubling or halving of the amount of enzyme gives twice or half of the original signal, respectively. The simplest way to determine the linear range is to select your preferred assay time (e.g. 30 min) and temperature (e.g. 23°C), which leaves only one further parameter to be investigated, the dilution of enzyme. The linear range is evident from a plot of absorbance versus enzyme dilution. If the absorbance value of any sample exceeds 2 it is usually necessary to dilute the enzyme further. For more guidance on assay design and the reasons for non-linearity in assays please refer to the relevant technical guides on our website.

2.2 Avoiding phosphate contamination

Since the assay measures P_i released from ATP, any free P_i present in the assay components *before* the reaction starts will give rise to a background signal.

The most common source of free P_i is the enzyme sample itself. Please note that all crude extracts of tissues and cells will contain free phosphate unless steps have first been taken to remove it.

To check if your enzyme sample contains free P_i, mix the assay components shown in Table 3.

Table 3. Checking for P_i contamination

Solution	Enzyme (µl)	MQ Water (µl)	0.1mM P_i (µl)
1	100	100	0
2	100	60	40
3	0	160	40
4	0	200	0

Ideally 2 & 3 will give high signals and 1 & 4 will give low signals. If the signal for sample 1 is relatively high compared with that for sample 4, your enzyme is contaminated. Free P_i in the enzyme may be eliminated by dialysis or desalting. Alternatively, P_iBind™ resin may be used (see 'Related products').

Note: Phosphate buffer should not be used to prepare tissue or cell homogenates.

2.3 Using blanks correctly

In general, you should set up assay blanks (i.e. lacking enzyme) and subtract the average blank value from all other wells before you do any calculations with the assay data e.g. calculating the amount of phosphate generated by reference to the standard curve (Section 2.5).

2.4 Effects of some common substances

Table 4 lists chemicals that are often used in enzyme assays, with the expected type of interference (if any) for the concentrations stated below.

Table 4. Effects of some common assay components

Component	Conc.*	Effect
NaCl	250mM	None
KCl	250mM	None
$MgCl_2$	25mM	None
DTT	0.25mM	Slight signal loss
b-ME	0.5mM	None
Tris	25mM	None
HEPES	25mM	None
MES	25mM	None
MOPS	25mM	None
BSA	0.1mg/ml	None
BSA	1mg/ml	Risk of precipitation
DMSO	2.5%	None
Detergents	0.03%	See footnote**

*The stated values refer to concentrations in the assay samples **before** the addition of PiColorLock™ mix.
**Very low concentrations of detergent (0.002-0.005%) may cause precipitation. If a detergent is needed, use a concentration of >0.03%. Tween 20 is usually a good choice.

2.5 Standard curves

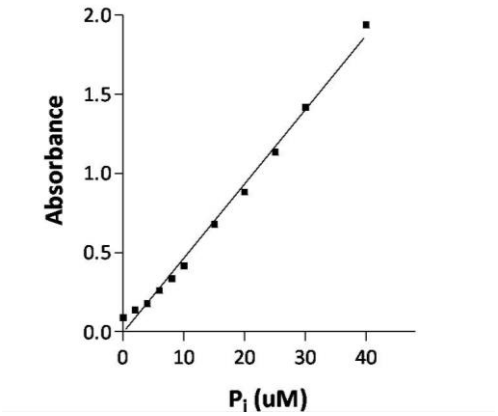
A standard curve is required if you wish to calculate enzyme activity. Prepare a set of P_i standards using the 0.1mM P_i stock (see Table 5). Set up duplicate wells containing 200µl of each standard and add 50µl of P_iColorLock™ mix. Two minutes later add 20µl of Stabilizer, and ***mix thoroughly***. After 30 minutes, read the plate. Subtract the blank values (i.e. for sample 12) and plot absorbance versus concentration of P_i.

Table 5. Phosphate standards

Tube	0.1mM P_i standard (µl)	MQ Water (µl)	Concentration of P_i (µM)
1	500	500	50
2	450	550	45
3	400	600	40
4	350	650	35
5	300	700	30
6	250	750	25
7	200	800	20
8	150	850	15
9	100	900	10
10	50	950	5
11	25	975	2.5
12	0	1000	0

A typical standard curve is shown in Fig 2. The absorbance value for samples is used to determine µM P_i (i.e. from the intercept on the x-axis of the standard curve).

Figure 2. P_i standard curve



2.6 Calculating enzyme activity

If you need to do this, one unit is the amount of enzyme that catalyses the reaction of 1 µmol of substrate per minute. The activity (units/ml) of your *undiluted* enzyme sample is given by the equation:
Activity = (AxC)/500B, Where,
A = concentration of P_i (uM) determined from the standard curve.
B = assay time is in minutes.
C = enzyme dilution factor (Note: the value for 'C' *must* be 1 or >1; e.g. C= 100 for a 1/100 dilution of enzyme).
Note: The above equation is valid ***only*** if the assay is set up as indicated in the protocol (i.e. the assay volume is 200µl, comprising 100µl of enzyme and 100µl of substrate mix).

RELATED PRODUCTS

Description		Prod. Code
Ultra-purified freeze dried ATP. Designed to give the lowest possible backgrounds.	10mM lyophilized ATP	601-9999
The detection reagent from this kit is available separately and can be used to assay any P_i-generating enzyme.	P_iColorLock™	303-0125
PiBind™ resin can be used to quickly remove Pi from water, buffers and protein samples.	P_iBind™ resin	501-0015

FAQS

Why do I get a high background when my enzyme definitely has no free P_i?

This is almost certainly caused by inadequate mixing of the stabilizer. This results in a high background signal because of non-enzymatic decay of ATP substrate. The stabilizer is added in a relatively small volume (20µl), and the operation of pipetting up and down with a pipette set to 20µl volume may not result in sufficient mixing when the total volume is 270µl. Try pipetting up and down while stirring at the same time. Alternatively, add the stabilizer with one pipette set at 20µl volume and mix using a larger pipette set to ~150µl volume. This ensures thorough mixing of the stabilizer solution with minimal effort.

For additional information please see our FAQs page: https://www.expedeon.com/faqs

TECHNICAL SUPPORT

For technical enquiries get in touch with our technical support team at: technical.enquiries@expedeon.com

For further information see our website: www.expedeon.com