

Version 2n Last updated 22 July 2021

# ab270551 ATPase Assay Kit

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Applicable to Expedeon product codes: 601-0120

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ATPase Assay Kit datasheet:

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For measuring the activity of ATPase enzymes in microplates.

This product is for research use only and is not intended for diagnostic use.

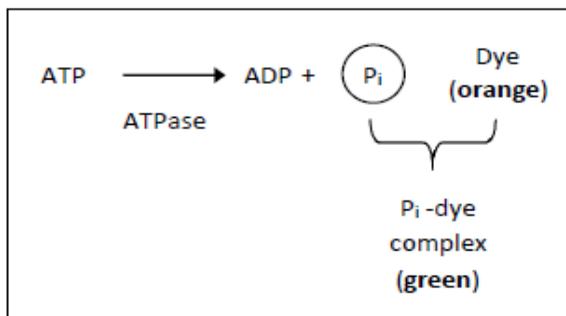
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### 1. Overview

ATPase Assay Kit (ab270551) 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 nonenzymatic ATP hydrolysis. The PiColorLock™ assay is based on the change in absorbance of the dye malachite green in the presence of phosphomolybdate complexes. Unlike most malachite dye-based solutions,

PiColorLock gives a stable end-point signal and is not prone to precipitation. PiColorLock™ has special additives to prevent backgrounds arising from nonenzymatic ATP hydrolysis.



Principle of the ATPase assay kit.

## 2. Materials Supplied and Storage

Store kit at 4°C immediately on receipt and check below for storage for individual components. Kit can be stored for 1 year from receipt, if components have not been reconstituted.

Avoid repeated freeze-thaws of reagents.

Item	192 assays	Storage temperature
PiColorLock™	1 x 10 mL	4°C
Accelerator	1 x 0.25 mL	4°C
Stabilizer	1 x 5 mL	4°C
0.1 M Magnesium chloride	1 x 1.5 mL	4°C
0.5 M Tris pH 7.5	1 x 5 mL	4°C
0.1 mM Pi Standard	1x 5 mL	4°C
ATP (lyophilized)	4 vials	-20°C or -70°C
96-well plates	2 plates	RT

***Δ Note:*** Caution - PiColorLock™ is very acidic.

### 3. Technical Considerations

#### 3.1 Checking for Pi contamination

Since the assay measures Pi released from ATP, any free Pi present in the assay components before the reaction starts will give rise to a background signal. The most common source of free Pi is the enzyme sample itself.

***Δ Note:*** 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 Pi, mix the assay components shown in the table , below.

Solution	Enzyme (μL)	MilliQ water (μL)	0.1 mM Pi (μL)
1	100	100	0
2	100	60	40
3	0	160	40
4	0	200	0

***Δ Note:*** Ideally 2 and 3 will give high signals and 1 and 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 Pi in the enzyme may be eliminated by dialysis or desalting.

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

To remove free Pi from the sample, please check the Inorganic Phosphate Binding Resin (ab270547).

### 3.2 Effects of some common substances:

Component	Concentration*	Effect
NaCl	250 mM	None
KCl	250 mM	None
MgCl <sub>2</sub>	25 mM	None
DTT	0.25 mM	Slight signal loss
β-ME	0.5 mM	None
Tris	25 mM	None
HEPES	25 mM	None
MES	25 mM	None
MOPS	25 mM	None
BSA	0.1 mg/mL	None
BSA	1.0 mg/mL	Risk of precipitation
DMSO	2.5%	None
Detergents	0.03%	See below**

**Δ Note:** \*The stated values refer to concentrations in the assay samples before the addition of PiColorLock™ mix.

**Δ Note:** \*\* 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.

### 3.3 How to determine assay conditions:

1.1. 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 mins and 1 hour before addition of the Pi-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 mins) 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.

### 3.4 Blanks

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.

## 4. Reagent Preparation

### 4.1 ATP substrate:

Reconstitute the lyophilized ATP to 10 mM concentration by adding the volume of MQ H<sub>2</sub>O stated on the vial label. 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.

**Δ Note:** *The reconstitution volume is batch specific.*

### 4.2 Substrate/ buffer mix:

The assay kit is supplied with 0.5 M 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<sup>2+</sup> (0.1 M MgCl<sub>2</sub> is supplied with the kit) but again you can substitute your own metal ion, or include other metal ions, as required.

The table below shows the volumes required to make up substrate / buffer mix for selected numbers of wells. The ATP concentration in this mix is 1 mM. Once the enzyme has been added to the substrate/ buffer mix, the final buffer, Mg<sup>2+</sup> and

ATP concentrations are 50 mM, 2.5 mM, and 0.5 mM, respectively.

No. of wells	0.5 M Buffer ( $\mu\text{L}$ )	0.1 M $\text{MgCl}_2$ ( $\mu\text{L}$ )	10 mM ATP ( $\mu\text{L}$ )	MilliQ water ( $\mu\text{L}$ )
1	20	5	10	65
25	500	125	250	1,625
50	1,000	250	500	3,250
75	1,500	375	750	4,875
100	2,000	500	1,000	6,500
150	3,000	750	1,500	9,750
200	4,000	1,000	2,000	13,000
250	5,000	1,250	2,500	16,250
500	10,000	2,500	5,000	32,500

#### 4.3 PiColorLock™ mix:

Prepare the mix immediately before the reagent is required by adding 1/100 volume of Accelerator to PiColorLock™ .

The table below gives the volumes of reagent required for the specified numbers of wells.

No. of wells	PiColorLock™ (μL)	Accelerator (μL)
1	50	0.5
25	1,250	12.5
50	2,500	25
75	3,750	37.5
100	5,000	50
150	7,500	75
200	10,000	100
250	12,500	125
500	25,000	250

*Δ Note: The PiColorLock™ mix cannot be stored for long periods; make up only what you will use on the day.*

### 5. Assay Procedure:

- Equilibrate all materials and prepared reagents to room temperature just prior to use and gently agitate.

5.1 Make substrate/buffer mix.

5.2 Make PiColorLock™ mix by adding Accelerator to PiColorLock™ reagent just before use.

5.3 Set up assays with your ATPase; add 100μL enzyme to 100μL substrate/buffer mix.

5.4 Incubate for a fixed time at set temperature.

5.5 Add 50 μL of PiColorLock™ mix to stop reactions.

- 5.6 After 2 minutes, add 20  $\mu\text{L}$  of Stabilizer and mix thoroughly by pipetting up and down.
- 5.7 After 30 mins, read the plate at a wavelength in the range 590-660 nm

## 6. Standard Curve

A standard curve is required if you wish to calculate enzyme activity.

- Prepare a set of Pi standards using the 0.1 mM Pi stock (see the table, below).
- Set up duplicate wells containing 200  $\mu\text{L}$  of each standard and add 50  $\mu\text{L}$  of PiColorLock™ mix.
- Two minutes later add 20  $\mu\text{L}$  of Stabilizer and mix thoroughly.
- After 30 mins, read the plate. Subtract the blank values (i.e. for sample 12) and plot absorbance versus concentration of Pi.

Tube #	0.1 mM Pi Standard ( $\mu\text{L}$ )	MilliQ water ( $\mu\text{L}$ )	Concentration of Pi ( $\mu\text{M}$ )
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	1,000	0

## 7. Enzyme Activity Calculation:

If you need to do this, one unit is the amount of enzyme that catalyzes the reaction of 1  $\mu\text{mol}$  of substrate per minute. The activity (units/ml) of your undiluted enzyme sample is given by the equation:

$$\text{Activity} = (\text{AxC})/500\text{B}$$

Where:

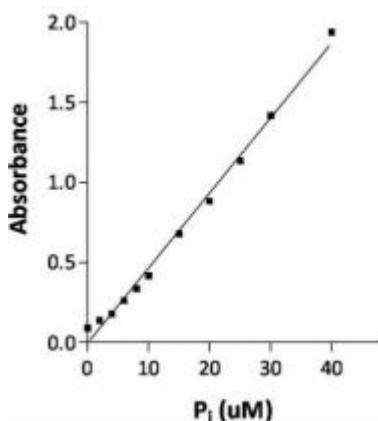
A = concentration of Pi ( $\mu\text{M}$ ) determined from the standard curve (see Section 6).

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).

*$\Delta$  Note: The above equation is valid only if the assay is set up as indicated in the protocol (i.e. the assay volume is 200 $\mu\text{l}$ , comprising 100 $\mu\text{l}$  of enzyme and 100 $\mu\text{l}$  of substrate mix).*

## 8. Typical Data



**Figure 1:** Standard Curve for High Throughput GTPase Assay Kit - PiColorLock™

## 9. FAQ

### 1. Why do I get a high background when my enzyme definitely has no free Pi?

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 (20  $\mu$ L), and the operation of pipetting up and down with a pipette set to 20  $\mu$ L volume may not result in sufficient mixing when the total volume is 270  $\mu$ L. Try pipetting up and down while stirring at the same time. Alternatively, add the stabilizer with one pipette set at 20  $\mu$ L volume and mix using a larger pipette set to  $\sim$ 150  $\mu$ L volume. This ensures thorough mixing of the stabilizer solution with minimal effort.

### 2. I would like to measure the conversion of pyrophosphate to phosphate. Can I use the PiColorLock™ Phosphate Detection System for this purpose?

Yes, only the phosphate will give a signal, pyrophosphate will not.

### 3. I have 5% DMSO in my assay. Can I use PiColorLock™?

Yes, the reagent is designed for drug screening work and other situations that require DMSO.

### 4. I have phosphate in my enzyme. What can I do?

You can dialyse or desalt the enzyme into a phosphate-free buffer. Alternatively, you can use the Inorganic Phosphate Binding Resin (ab270547) resin to remove the phosphate.

# Technical Support

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