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ATPase assay kit

Product codes: 601-0120, 601-0121, 601-0122

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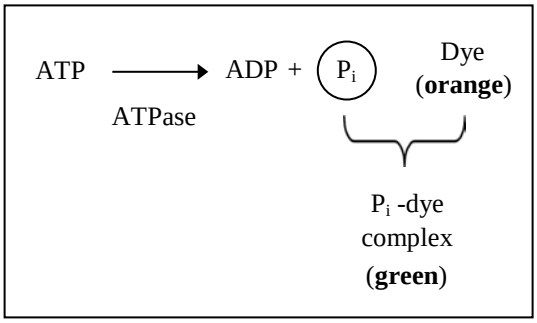
1. INTRODUCTION

The ATPase colorimetric assay kit employs a 96-well plate format with all reagents necessary for measuring ATPase activity. It also contains P_iColorLock™ Gold (an improved malachite green reagent) with special additives to prevent backgrounds arising out of non-enzymatic ATP hydrolysis.

Fig 1. Principle of the ATPase assay kit:



2. INSTRUCTIONS

2.1. Components in the 2-plate ATP assay kit (5-plate kit amounts in brackets)

Store at 4°C:

1 x 10ml of P_iColorLock™ Gold*	(1 x 25ml)
1 x 0.25ml of Accelerator	(1 x 0.5ml)
1 x 5ml of Stabiliser	(1 x 10ml)
1 x 1.5ml of 0.1M $MgCl_2$	(2 x 1.5ml)
1 x 5ml of 0.5M Tris pH 7.5	(1 x 10ml)
1 x 5ml of 0.1mM P_i standard	(1 x 10ml)

Note: Take care, the solution is very acidic*

Store at -20 or -70°C:

4 vials of lyophilised ATP	(10 vials)

Store at room temperature:

2 x 96-well plates	(5 plates)

2.2. Overview of ATP colorimetric assay

(a) Make substrate/buffer mix.

(b) Make 'Gold mix' by adding Accelerator to P_iColorLock™ Gold reagent.

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

(d) Add 50ul of Gold mix to stop reactions.

(e) After 2 minutes, add 20ul of Stabiliser and mix by pipetting up and down.

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

2.3. Preparation of reagents

2.3.1. ATP substrate

ATP should be reconstituted by addition of 540ul of water. Keep the 10mM ATP on ice while in use and snap freeze any surplus reagent (avoiding multiple freeze-thaw cycles) and store at -70°C.

2.3.2. 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, if required.

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

Table 1. Preparation of SB mix

No. of Wells	0.5M Buffer (ul)	0.1M $MgCl_2$ (ul)	10mM ATP (ul)	Water (ul)
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

2.3.3. Supplied Assay Reagents

The P_iColorLock™ Gold reagent should be used direct from the fridge. All other reagents should be warmed to room temperature before use.

2.3.4. Gold mix

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

Table 2. Gold mix volumes.

No. of Wells	P_iColorLock™ Gold (ul)	Accelerator (ul)
10	500	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

2.4. Assay procedure

The procedure involves mixing 100ul of SB mix with 100ul of appropriately diluted enzyme and incubating for a fixed period of time at a set temperature. The reaction is then stopped by adding 50ul of Gold mix. After 2 minutes, the stabiliser is added and mixed in thoroughly by pipetting up and down. A green colour develops for samples with Pi. The colour reaches a maximum at 30 minutes and the plate can then be read at any wavelength from 590-660 nm.

2.4.1. How to determine assay conditions

There are no strict rules on enzyme dilution and assay time, as these parameters will depend on the activity of the particular enzyme, the concentration of the enzyme, and operator preferences with regard to assay time and temperature. In general assays are usually between 15min and 1 hour. Plate assays are most easily run at room temperature. Once established, the assay conditions (ideally) should not be changed as data from different experiments can be more easily compared.

One important point is to operate in the 'linear range' i.e. under conditions where doubling or halving of the amount of enzyme will give exactly twice or half the signal, respectively. The simplest way to find the linear range is to pick an assay time (e.g. 30min) and temperature, which then leaves only one variable, the dilution of enzyme. The enzyme is tested at several dilutions and from a graphical plot of signal versus enzyme concentration the linear range will be obvious. Broadly speaking, if

the absorbance value of a sample exceeds 2, you probably should be diluting the enzyme further. For more guidance on assay set up and the reasons for non-linearity please refer to the enzyme guide, available on www.innovabiosciences.com

2.4.2 Avoiding phosphate contamination

Since the assay measures P_i 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. Please note that all crude extracts of tissues and cells will contain free phosphate, unless steps are first 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 (ul)	Water (ul)	0.1mM P_i (ul)
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 and 4 give low signals. If the signal for sample 1 is relatively high compared with that for 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 or be used as an assay buffer.

2.4.3. Using blanks correctly

You should always set up assay blanks (i.e. lacking enzyme) and subtract the average blank value from all other wells before you do any other subtractions or other calculations on the data.

2.4.4. 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 stated concentrations.

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 Gold 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 a good choice.

2.4.5. Standard curves

A standard curve is required if you wish to calculate enzyme activity. Prepare a set of

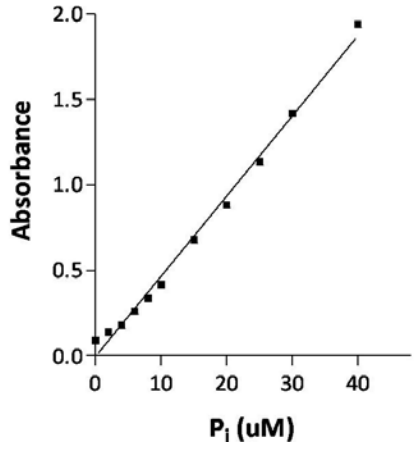
P_i standards using the 0.1mM P_i stock (see Table 5). Set up duplicate wells containing 200ul of each standard and add 50ul of Gold mix. Two minutes later add 20ul of Stabiliser, and mix thoroughly. After 30 minutes, read the plate. Subtract the blank values (i.e. for sample 12) and plot absorbance versus concentration of P_i.

Table 5. Phosphate standards

Tube	0.1mM P_i standard (ul)	Water (ul)	Concentration of P_i (uM)
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 1. The absorbance value for samples is used to determine uM P_i (from the intercept on the x-axis of the standard curve).

Figure 1. P_i standard curve



2.4.6 . Calculating enzyme activity

If you need to do this, one unit is the amount of enzyme that catalyses the reaction of 1 umol 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 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).

The above equation is valid *only* if the assay is set up as indicated in the protocol (i.e. the assay volume is 200ul, comprising 100ul of enzyme and 100ul of substrate mix).

4. Related products

303-0125 P_iColorLock™ Gold

The detection reagent from this kit is available separately and can be used to assay any P_i-generating enzyme.

501-0015 P_iBind™ resin

P_iBind™ resin can be used to eliminate P_i from water, buffers and protein samples.

5. FAQ

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

This is most likely connected with inadequate mixing of the stabilizer, which results in a high background signal because of non-enzymatic decay of ATP substrate. As the stabiliser is added in a 20ul volume, the operation of pipetting up and down does not exchange much liquid at each cycle of pipetting. Try pipetting up and down and stirring at the same time. Alternatively, add the stabiliser with one pipette set at 20ul volume and mix using a larger pipette set to ~150ul volume. This is a very reliable method of mixing.

Technical support:
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