



ATPase assay kit (high sensitivity)

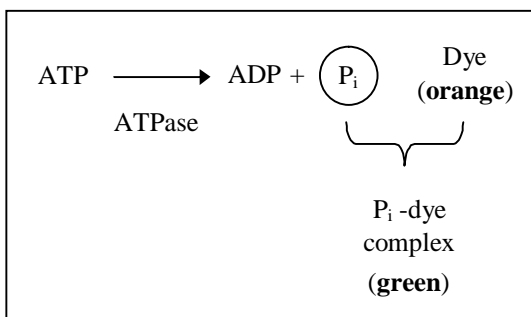
Release 007; July 2005

Technical bulletin 654

1. INTRODUCTION

The ATPase colorimetric assay kit employs a 96-well plate format with all reagents necessary for measuring ATPase activity. The kit contains specially purified P_i -free ATP to ensure the lowest possible background signals. It also contains P_i ColorLock Gold reagent (an improved malachite green formulation) with additives to prevent background signals arising out of non-enzymatic ATP hydrolysis. Assays can be read anywhere in the wavelength range 590-660nm.

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_i ColorLock 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) |

Store at -70°C:

| | |
|--------------------------------|-----------|
| 2 x 1ml specially purified ATP | (5 x 1ml) |
|--------------------------------|-----------|

Store at room temperature:

| | |
|--------------------|------------|
| 2 x 96-well plates | (5 plates) |
|--------------------|------------|

*Exercise caution – this reagent contains 5M HCl. Wear gloves and safety glasses.

2.2. Overview of ATP colorimetric assay

- Make substrate/buffer mix.
- Make 'Gold mix' by adding Accelerator to P_i ColorLock™ Gold.
- Set up assays with ATPase (not supplied); 100 μ l enzyme plus 100 μ l substrate.
- Add 50 μ l of Gold mix to stop reactions.
- After 2 minutes, add 20 μ l of stabiliser.
- 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 thawed out by warming between the fingers or by immersion in cold water. Store the substrate on wet ice until required. Surplus ATP stock should be frozen in aliquots and stored at -70°C.

2.3.2 Substrate/buffer (SB) mix

The assay kit is supplied with 0.5M Tris assay buffer pH 7.4. However, you may use any other non-phosphate-containing buffer if you wish. Most ATPases will require a metal ion cofactor. $MgCl_2$ (0.1M) is supplied with the kit but you can substitute your own metal ion, or include other metal ions, if required.

Table 1 (below) shows the volumes required to make up SB mix for selected numbers of wells. These volumes result in final buffer and Mg^{2+} concentrations (i.e. after your enzyme has been added) of 50mM and 2.5mM, respectively. If you require concentrations that are different to those suggested, simply alter the amount of water added in Table 1 to compensate. The final concentration of purified ATP is 0.5mM.

Table 1. Preparation of SB mix

| No of Wells | 0.5M Buffer (ml) | 0.1M MgCl ₂ (ml) | 10mM ATP (ml) | Water (ml) |
|-------------|------------------|-----------------------------|---------------|------------|
| 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. Gold mix

Prepare 'Gold mix' shortly before the reagent is required by adding 1/100 vol. of Accelerator to P_iColorLock™ Gold (see Table 2 below).

Table 2. Volumes needed to make Gold mix

| No of Wells | P _i ColorLock Gold (ml) | Accelerator (ml) |
|-------------|------------------------------------|------------------|
| 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 |

2.4. Important considerations**2.4.1 Checking for free P_i in enzyme preps**

Free P_i will cause a high background. To check if your enzyme sample contains free P_i, make up the solutions shown in Table 3. Set up duplicate wells (200µl/well) and add 50µl of Gold mix. Two minutes later add 20µl of Stabiliser. Solutions 1 & 4 should change colour from dull yellow into golden yellow over a period of 5-10 minutes. Solutions 2 & 3 should give a strong green colour as soon as the Gold mix is added. After 30 minutes, read the plate at 635nm (max. absorbance) or use a wavelength in the range 590-660nm (which will give at least 80% of the maximum signal). Solution 4 should give <0.15

absorbance units. If sample 1 gives a similar value (<0.2) P_i contamination is minimal. Solutions 2 & 3 will give >1.0 absorbance units.

Significant amounts of free P_i in the enzyme may be eliminated by dialysis or desalting. Alternatively, P_iBind™ resin may be used (see 'Related products').

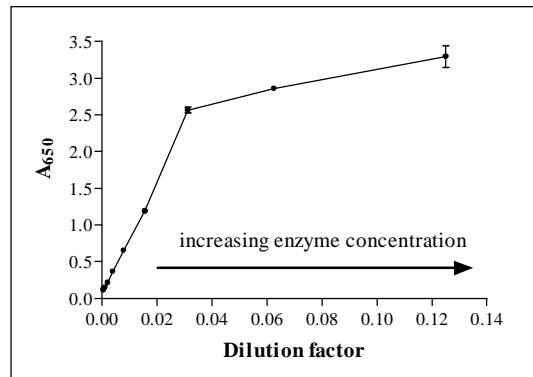
Table 3. Checking for P_i contamination

| Solution | Enzyme (ml) | Water (ml) | 0.1 mM P _i (ml) |
|----------|-------------|------------|----------------------------|
| 1 | 100 | 100 | 0 |
| 2 | 100 | 60 | 40 |
| 3 | 0 | 160 | 40 |
| 4 | 0 | 200 | 0 |

2.4.2 Absorbance versus amount of enzyme

It is important for quantitative work to ensure that absorbance versus enzyme concentration is linear. Most assays are linear if substrate hydrolysis is less than 15%. By fixing the assay time (e.g. 30 min) and temperature (e.g. 25°C) the degree of hydrolysis may be controlled simply by using a suitable dilution of enzyme.

Fig 2 below shows an illustrative plot of absorbance versus enzyme concentration.

Fig 2. Absorbance versus amount of enzyme

In this example, the assay is linear up to OD 2.5. A dilution factor of 0.02 would be ideal for assay work, as this gives a large signal (~1.5) and lies in the middle of the linear range. Calculations based on results for dilution factors between 0.04 and 0.12 (i.e. the region with reduced slope) will clearly underestimate the true level of enzyme activity. Appendix 1 gives a simple equation for calculating enzyme activity values.

2.4.3 Assay time

While almost any time can be used, it is usual to select a time between 15 and 60 min. If the assay is short, it is important to ensure that the reagents have equilibrated to the correct temperature before the assay is set up (see below).

2.4.4 Absorbance versus time

A linear relationship should be seen. Make sure that the enzyme and substrate have equilibrated to the required assay temperature before they are combined in the plate, otherwise there will be a lag while the reagents warm up in the first few minutes of the assay. The calculated amount of P_i generated per minute (which is used to determine activity) may be underestimated in this situation.

2.4.5 Compatible 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 in the ATPase assay

| Component | Conc. * | Effect |
|-------------------|-----------|-----------------------|
| Salts: | | |
| NaCl | 250 mM | None |
| KCl | 250 mM | None |
| MgCl ₂ | 25 mM | None |
| DTT | 0.25 mM | Slight signal loss |
| βME | 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 mg/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 in the assay, use at least 0.03%. Tween 20 is the preferred choice. SDS should be avoided, but since the Gold mix is very acidic there is no need to use SDS if the purpose is simply to stop the reaction.

2.5. Standard curves

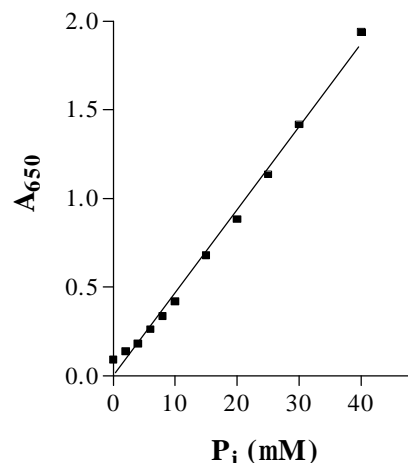
A standard curve is required if you wish to calculate enzyme activity. Prepare a set of P_i standards using the 0.1mM P_i stock (see Table 5). Set up duplicate wells containing 200μl of each standard and add 50μl of Gold mix. Two minutes later add 20μl of Stabiliser. After 30 minutes, read the plate.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

Table 5. Phosphate standards

| Tube | 0.1 mM P_i standard (ml) | Water (ml) | Concentration of P_i (mM) |
|------|----------------------------|------------|-----------------------------|
| 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 3. The absorbance value for the assay samples is used to determine μM P_i from the intercept on the x-axis of the standard curve.

Fig 3. Standard curve for P_i



3. Shelf life

If the components are stored correctly, optimum performance will be observed for >6 months. The Gold mix *cannot* be stored for long periods, make up only what you will use on the day.

4. Related products

#303-0030 Pi ColorLock™ Gold

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

#302-0125 Pi ColorLock™ ALS

This reagent is similar to PiColorLock Gold but the sample:Colorlock ratio is 1:4 rather than 4:1.

#501-0015 PiBind™ resin

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

5. Trouble shooting/FAQ

Q1. Why do assays go non-linear at high enzyme concentrations?

The rate of reaction is dependent on the concentration of substrate. If a large fraction of the substrate is utilised (i.e. at high enzyme concentrations) there may be a reduction in the rate. Hydrolysis of 5-15% of the substrate is usually fine.

Q2. Do I need to subtract blanks from the standard curve?

No, but we recommend it. The blank is the value obtained in the absence of P_i, and the value is ~0.1. Thus, if the OD for a sample is 1.0, the signal due to P_i released by the ATPase is 0.9. Remember, if you subtract blanks from your standard curve, you must also subtract blanks from your assay data (but see FAQ3)

Q3. What controls do I need?

You can simply omit the enzyme and replace with enzyme diluent. However, a more rigorous approach is to *include* the enzyme in the control, but change the order of addition so that the Gold mix is added to the substrate *before* the enzyme. This control should be set up when the other

assay wells are being stopped, so that all wells receive the Gold mix at the same time. The advantage of this approach is that subtraction of a single control value corrects for all free P_i, whatever its source. Moreover, this operation subtracts the blank value too, thus the resulting value can be used to determine P_i from the blank-subtracted standard curve.

Q4. Why am I getting high backgrounds when there is no free P_i in my enzyme or substrate?

Did you remember to add the Stabiliser? It is essential that the Stabiliser is added to prevent high backgrounds caused by non-enzymatic hydrolysis of ATP.

Q5. I know my sample has activity but all my wells are yellow. Why is this?

The most likely explanation is that the Stabiliser has been added with, or immediately following, the Gold mix. Make sure that the Stabiliser is added two minutes after the Gold mix.

Appendix I

Calculation of enzyme activity

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 following equation:

$$\text{Activity} = (A \times C)/500B$$

where, A = concentration of P_i (μM) determined from the standard curve

B = assay time in minutes

C = reciprocal of the enzyme dilution factor *

*Note: the value for 'C' *must* be 1 or >1. For example, if the enzyme is diluted 1/100 prior to the addition of the 100 μl enzyme sample to the substrate, C =100).

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). Note: the 1:1 dilution that occurs upon addition of the enzyme to the substrate has been factored into the above equation, so the only dilution factor you need to consider is that of the enzyme before the enzyme is added to the plate.