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601-0120 Protocol

Colorimetric ATPase Assay Kit Protocol (601-0120)

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 Pi -free ATP to ensure the lowest possible background signals. It also contains PiColorLock Gold reagent (an improved malachite green formulation) with additives to prevent background signals arising out of nonenzymatic ATP hydrolysis. Assays can be read anywhere in the wavelength range 590-660nm.

ATP ----ATPase----> ADP + [Pi and orange dye] ----> Pi- dye complex (green)

2. INSTRUCTIONS
2.1 Components in the 2-plate ATP assay kit
(5-plate kit amounts in brackets)
Store at 4 degrees Celsius:
1 x 10ml of PiColorLock Gold* (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)

Store at -70 degrees Celsius:
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
(a) Make substrate/buffer mix.
(b) Make Gold mix by adding Accelerator to PiColorLock Gold.
(c) Set up assays with ATPase (not supplied); 100ul enzyme plus 100ul substrate.
(d) Add 50 ul of Gold mix to stop reactions.
(e) After 2 minutes, add 20ul of stabilizer.
(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 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 degrees Celsius.

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. MgCl2 (0.1M) is supplied with the kit but you can substitute your own metal ion, or include other metal ions, if required. The examples below show the volumes required to make up SB mix for selected numbers of wells. These volumes result in final buffer and Mg2+ 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.

Preparation of SB mix
1 well --> 20ul of 0.5M Buffer --> 5 ul of 0.1M MgCl2 --> 10ul of 10mM ATP --> 65ul of Water
25 wells --> 500ul of 0.5M Buffer --> 125 ul of 0.1M MgCl2 --> 250ul of 10mM ATP --> 1625ul of Water

(Use these same ratios for any number of wells.)
2.3.3. Gold mix
Prepare Gold mix shortly before the reagent is required by adding 1/100 vol. of Accelerator to PiColorLock Gold (see examples below).

Table 2. Volumes needed to make Gold mix
1 well --> 50ul of PiColorLock Gold --> 0.5ul of Accelerator
25 wells --> 1250ul of PiColorLock Gold --> 12.5ul of Accelerator

(Use these same ratior for any number of wells.)

2.4. Important considerations

2.4.1 Checking for free Pi in enzyme preps
Free Pi will cause a high background. To check if your enzyme sample contains free Pi, make up the solutions shown in the examples below. Set up duplicate wells (200ul/well) and add 50ul of Gold mix. Two minutes later add 20ul of Stabilizer. Solutions 1 & 4 should change color from dull yellow into golden yellow over a period of 5-10 minutes. Solutions 2 & 3 should give a strong green color 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) Pi contamination is minimal. Solutions 2 & 3 will give >1.0 absorbance units. Significant amounts of free Pi in the enzyme may be eliminated by dialysis or desalting. Alternatively, PiBind resin may be used (see 'Related products').

Table 3. Checking for Pi contamination
Solution #1: 100ul of Enzyme --> 100ul of Water --> 0ul of 0.1mM Pi
Solution #2: 100ul of Enzyme --> 60ul of Water --> 40ul of 0.1mM Pi
Solution #3: 0ul of Enzyme --> 160ul of Water --> 40ul of 0.1mM Pi
Solution #4: 0ul of Enzyme --> 200ul of Water --> 0ul of 0.1mM Pi

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 degrees Celsius) the degree of hydrolysis may be controlled simply by using a suitable dilution of enzyme. For example, if 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 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 Pi generated per minute (which is used to determine activity) may be underestimated in this situation.

2.4.5 Compatible substances
Below is a list of chemicals that are often used in enzyme assays, with the expected type of interference (if any) for the stated concentrations.

Effects of some common assay components in the ATPase assay:

Salts:
NaCl --> 250 mM --> No Effect
KCl --> 250 mM --> No Effect
MgCl2 --> 25 mM --> No Effect
DTT --> 0.25 mM Slight signal loss
bME --> 0.5 mM --> No Effect
Tris --> 25 mM --> No Effect
Hepes --> 25 mM --> No Effect
Mes --> 25 mM --> No Effect
Mops --> 25 mM --> No Effect

BSA --> 0.1 mg/ml --> No Effect
BSA --> 1 mg/ml Risk of Precipitation
DMSO --> 2.5% --> No Effect
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 Pi standards using the 0.1mM Pi stock (see below). Set up duplicate wells containing 200ul of each standard and add 50ul of Gold mix. Two minutes later add 20ul of Stabilizer. After 30 minutes, read the plate. Subtract the blank values (i.e. for sample 12) and plot absorbance versus concentration of Pi.

Phosphate standards
#1: 500ul of 0.1mM Pi standard --> 500ul of Water --> 50uM Concentration of Pi
#2: 450ul of 0.1mM Pi standard --> 550ul of Water --> 45uM Concentration of Pi
#3: 400ul of 0.1mM Pi standard --> 600ul of Water --> 40uM Concentration of Pi
#4: 350ul of 0.1mM Pi standard --> 650ul of Water --> 35uM Concentration of Pi
#5: 300ul of 0.1mM Pi standard --> 700ul of Water --> 30uM Concentration of Pi
#6: 250ul of 0.1mM Pi standard --> 750ul of Water --> 25uM Concentration of Pi
#7: 200ul of 0.1mM Pi standard --> 800ul of Water --> 20uM Concentration of Pi
#8: 150ul of 0.1mM Pi standard --> 850ul of Water --> 15uM Concentration of Pi
#9: 100ul of 0.1mM Pi standard --> 900ul of Water --> 10uM Concentration of Pi
#10: 50ul of 0.1mM Pi standard --> 950ul of Water --> 5uM Concentration of Pi
#11: 25ul of 0.1mM Pi standard --> 975ul of Water --> 2.5uM Concentration of Pi
#12: 0ul of 0.1mM Pi standard --> 1000ul of Water --> 0uM Concentration of Pi

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.