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ab270004 Phosphate Assay Kit - PiColorLock™

A product of Expedeon, an Abcam company

Applicable to Expedeon product codes 303-0030, 303-0125.

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Phosphate Assay Kit- PiColorLock™ datasheet:
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For measuring the activity of inorganic phosphate-generating enzymes in microplates.

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

Table of Contents

1. Overview

Phosphatases, ATPases and several other enzymes catalyze reactions in which inorganic phosphate (Pi) is released from an organic phosphorylated substrate. Phosphate Assay Kit-PiColorLock™ (ab270004) utilizes a specially formulated reagent to give sensitive detection of Pi and provides an alternative to the more hazardous radioactive methods and other less sensitive colorimetric assays. The reagent is compatible with DMSO, the solvent most commonly used in high throughput screening (HTS) applications.

The PiColorLock™ assay is based on the change in absorbance of the dye malachite green in the presence of phosphomolybdate complexes (see Figure 1). Unlike most malachite dye-based solutions, PiColorLock gives a stable end-point signal and is not prone to precipitation. Moreover, a special stabilizer ensures that the reagent can be used with acid labile substrates.

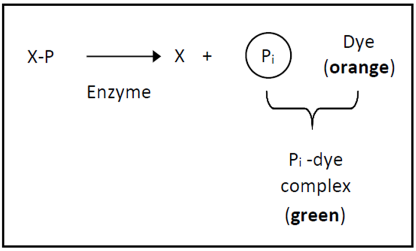

Figure 1: Principle of the PiColorLock™ assay

The kit comes in 2 sizes:

- 600 test (or 1500 assays in 384-well plates);
- 2500 test (or 6250 assays in 384-well plates).

ΔNote: *The reagent is sold in terms of volume and the quoted assay points shown are based on 200 µL sample volumes in 96-well plates (i.e. 50 µL mix/20 µL stabilizer added) and 80 µl sample volumes in 384-well plates (20 µL mix/8 µL stabilizer). If your assays are carried out in smaller volumes the number of assay points per bottle will obviously be greater than those stated here.*

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	600 test	2500 test	Storage temperature
PiColorLock™	30 mL	125 mL	+4°C
Pi Standard	1 Bottle	1 Bottle	+4°C
Stabiliser	1 Bottle	1 Bottle	+4°C
Accelerator	1 Vial	2 Vials	+4°C

Δ Note: *Caution - PiColorLockTM is very acidic.*

3. Reagent Preparation

3.1 Preparation of mix:

- PiColorLock™ reagent is supplied with a color accelerator and a special stabiliser to prevent high background signals with acid-labile substrates.
- Prepare 'mix' shortly before the reagent is required by adding 1/100 vol. of Accelerator to the PiColorLock™ reagent (e.g. for 10 mL of PiColorLock reagent add 0.1 mL of Accelerator).
- The mix is added to Pi-containing samples in a volume ratio of 1:4 (i.e 25% of the initial assay volume is added).

ΔNote: *While the PiColorLock™ reagent and accelerator are stable separately for months, the mix should not be stored for long periods. We recommend that you prepare quantities of mix that you are likely to use the same day.*

ΔNote: *The Stabilizer is always added to the assay plate last, and it is always added on its own. Never add the stabilizer directly to the mix. The volume of stabilizer needed is 0.1 vol. with respect to the initial assay volume (i.e.10% of the initial assay volume is added), and must be mixed thoroughly after addition.*

ΔNote: *As with any absorbance assay, absorbance is proportional to path length (i.e. depth of liquid in a microplate assay). Thus for a fixed type of assay well, an increase in volume will increase assay sensitivity. Please note, however, that the detection reagent is acidic and the assay wells must be able to accommodate the stop/detection reagent without risk of spilling.*

4. Assay Procedure

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

4.1 Preliminary checks for each new assay:

- For simplicity, an assay is considered to have three components: enzyme (E), substrate (S) and buffer (B). While this three-component system may not exactly reflect the components added to your particular assay, the basic checking procedure remains the same even if your assay has other additions.

4.2 Checking for Pi contamination:

- Assay reagents and buffers that contain free Pi can give rise to an unacceptable assay background.
- To check for contamination of reagents with free Pi prepare the solutions shown in the Table 1. You will need concentrates of E, S & B, but the solutions can be of any strength as long as the final concentration in each case is correct (i.e. single strength, 1x).

Table 1. Solutions required for checks on Pi contamination and substrate stability.

Solution number	Composition
1	B + S (no enzyme)
2	B + E (no substrate)
3	B (no enzyme, no substrate)
4	Pi-free water

ΔNote: *Where a component has been omitted the volume is made up with Pi-free water. 1 mL of each solution above is required to carry out the preliminary checks.*

- Add an appropriate volume (see below) of each of the solutions 1 through 4 in duplicate to a microplate followed by 0.25 volumes of mix (section 3.1). After 5 minutes (it is important to wait 5 minutes) add 0.1 volumes of stabilizer (i.e. 10% of the volume of the assay prior to the addition of mix) and ensure thorough mixing using a pipette set above 100 µL. For example, if the assay volume is 200 µL, add 50 µL of mix and, five minutes later, 20 µL of the Stabilizer, then mix with the pipette.
- The volume of solutions 1 through 4 added to the wells does not need to be 200 µL, as was given in the example above, but it should be the same as the volume of the enzyme-catalyzed reaction that you intend to set up. The conditions employed for the preliminary checks are then comparable with those of subsequent assays. Read the plate after 30 minutes using a wavelength in the range 590-660 nm (see section 4.5).
- The depth of solution in the well and the precise wavelength used will influence the absorbance values obtained, but none of the values should exceed 0.2 absorbance units. Often the values will be significantly below 0.2.
- If the value for Solution 1 (B+S) > Solution 3 (B alone), the stock substrate is almost certainly contaminated with free Pi.
- If the value for solution 2 (B+E) > solution 3 (B alone), the enzyme may need to be desalted or dialysed prior to use.
- If the value for solution 3 (B alone) > solution 4 (water), the buffer mixture itself may contain phosphate (do not use PBS!).

  ΔNote: *Any small differences among the four solutions are unimportant if all of the values are below 0.2 absorbance units.*

  ΔNote: *If any of the values are above 0.2, you may still be able to run the assay without modification by simply including appropriate control wells, particularly if you have a large assay window and the background is relatively small.*

4.3 Acid stability of the substrate:

- If you wish to examine whether your substrate is unstable in acid, set up wells as described in section 4.2 and add water instead of the stabilizer.
- Acid-labile substrates will give a rising background signal as the levels of Pi increase through non-enzymatic release. Some phosphorylated substrates will degrade rapidly (e.g. ATP) in the absence of the stabilizer while others will degrade more slowly (e.g. ser/thr phosphopeptides). AMP (as used in nucleotidase assays) is completely stable in mix.

  If you have a relatively stable substrate, you may be able to avoid the need for the Stabilizer by counting at an early time point (e.g. 30 or 60 minutes after mix is added).

4.4 Types of interference:

- The interference you are most likely to see is Pi contamination of substrates, though this is easily addressed by inclusion of suitable controls. Make sure substrates are stored at the correct temperature.
- Other types of interference are less common. The Table 2 lists chemicals that are often used in enzyme assays, with the expected type of interference (if any), and the acceptable range of concentrations for these reagents when tested individually.

Table 2. Effects of some common assay components in the PiColorLock™ assay

Component	Conconcentration*	Effect
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	1mg/mL	Precipitation
DMSO	2.50%	None
Detergents	0.03%	See footnote**

ΔNotes:

**The stated values refer to concentrations in the assay samples <u>before</u> the addition of mix.*

***Some detergents can, under certain conditions, accelerate precipitation of dye complexes and limit the time period over which plates can be counted. In general, very low concentrations of detergent are more likely to cause interference than higher concentrations. Use 0.03% or higher if a detergent is needed in the assay.*

Tween 20 is a preferred detergent and is very unlikely to cause interference. Triton X-100 is more likely to cause interference, though *this will depend on the other assay components to some extent; SDS, which is often used as a stop reagent, is very likely to cause precipitation and should not be used. Since the mix is very acidic there is no need to add SDS to assays if the only purpose of this addition is to stop the enzyme catalyzed reaction.*

4.5 Wavelength:

- The maximum signal for the PiColorLock™ reagent with Pi is obtained at ~635 nm but it is possible to achieve high sensitivity (>80% of the A635 value) over a broad range of wavelengths (590-660 nm). Many plate readers are supplied with a filter within this range. If your machine is not equipped with a suitable filter, please consult your instrument manufacturer.

4.6 Rate of colour development:

- In most dye-based assays for Pi the plates are counted soon after the addition of the detection reagent, because of the high risk of precipitation of the Pi-dye complex or because of rising background signals. With PiColorLock™, you should generally wait 30 minutes before counting the plate, but the reading can be taken many hours later if required.
- Absorbance values after 5, 30, 60 and 120 minutes typically are 90%, 96%, 98% and 99% of the ultimate end-point value, respectively.

5. Standard curves

5.1 General considerations:

- If relative absorbance values are more important than absolute values, as is usually the case in drug screening applications, it is probably not necessary to set up a standard curve. On the other hand, if the amount of Pi needs to be accurately quantified (e.g. to calculate enzyme activity) a standard curve will be needed and contamination of assay components with free Pi will need to be checked (Section 4.2) and, if required, suitable corrections applied to the assay data.

5.2 Preparation of standard curve:

- Prepare a set of Pi standards using the 0.1 mM Pi stock provided with the PiColorLock™ reagent, as indicated in Table 3. Set up triplicate wells of each standard.
- The volume of Pi standard added should be the same as the volume of the enzyme-catalyzed reaction that you propose to run, so that the depth of solution for both the standards and the assay samples is identical.
- Add 0.25 volumes of mix (section 3.1) to each well followed five minutes later by 0.1 volumes of stabilizer (i.e. 10% of the volume of the assay prior to addition of mix). Allow sufficient time for the color to develop (Section 4.5) before counting plates in the range 590-660nm (Section 4.6).
- Plot absorbance values versus concentration of Pi. Figure 1 in typical Data (section 6) shows an example calibration curve that was obtained using a clear 96-well plate with 200 µL standard + 50 µL mix and 20 µL of stabilizer read after 30 minutes at A650.

ΔNote: *The standards are designed to accommodate a broad range of assay situations so some of the concentrations in Table 3 may not be relevant to your particular assay. You may of course make other concentrations to customize your standard curve if required.*

Table 3. Phosphate standards.

Tube #	0.1mM Pi standard (µL)	MilliQ water (µL)	Concentration of Pi (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

6. Calculation of enzyme units:

There is much confusion over enzyme units largely because there is no single definition of the enzyme unit, and because standard curves can be plotted using concentration (e.g. µM, mM, or M) or absolute amount (e.g. nmol) on the x-axis. For the purposes of this discussion we assume that one unit is the amount of enzyme that converts 1 nmol of substrate into product per min, and we recommend that the standard curve is plotted using µM concentrations. You can plot absolute amounts instead if you prefer but at some point, in the calculations nmol per ml will have to be determined, thus it is generally easier to start with concentrations.

The aim is to determine the number of enzyme units per mL of undiluted enzyme stock. The required steps are shown below:

1. Determine the concentration of Pi arising from the enzyme catalyzed reaction. This is determined from the measured absorbance value (after correction for any contaminating Pi, see 7.4) by reference to the standard curve. The value on the x-axis is a µM value (i.e. nmol Pi/mL). While your assay volume is less than 1 mL (if you are running a microplate assay), this is irrelevant as far as concentration is concerned.

2. Divide by the assay time in minutes (for the enzyme-catalyzed reaction). This step gives nmol of Pi generated per min per mL, which as you can see from the above unit definition is the number of enzyme units per mL (= enzyme activity).

ΔNote: *If your reaction generates more than one Pi molecule per molecule of substrate hydrolyzed your activity value will need to be corrected. For example, pyrophosphatases act on PPi and the apparent activity is double the true activity because two molecules of product are generated for each molecule of substrate consumed.*

3A. Since the enzyme must have been diluted upon its addition to the other assay components, multiply the above result by the total volume of your assay (i.e. prior to addition of mix) and divide by the volume of the enzyme addition e.g. If you added 5 μL of enzyme to an assay that was 50 μL in total (prior to the addition of mix), multiply the result from step 2 by 10.

3B. If the stock enzyme was diluted down prior to its addition to the assay multiply the result from step 3A by the dilution factor, e.g. if you used a 1/100 dilution of enzyme you need to multiply by 100.

Steps 3A and 3B are correct for all the enzyme dilution factors and give the number of enzyme units per mL of undiluted enzyme stock.

Finally, if you need to calculate specific activity (= enzyme units per mg) divide the above result by the number of mg of protein per mL of undiluted enzyme stock.

7. Typical Data

Data provided **for demonstration purposes only**.

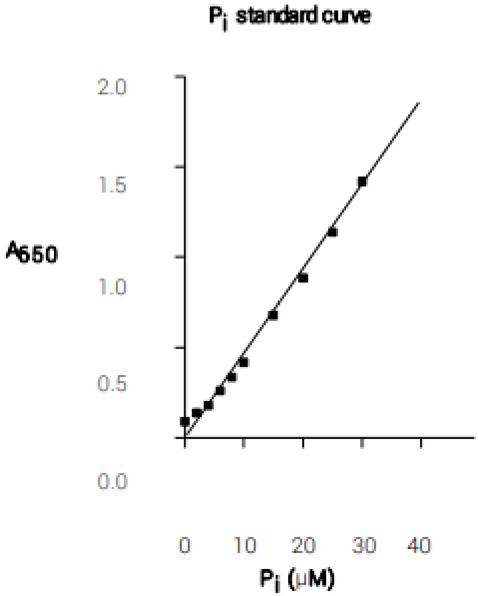

Figure 1: Standard curve for PicolorLock™

8. Troubleshooting

8.1 I have a high background but cannot seem to isolate the source of the problem.

Detergents used in glass washers may contain high concentrations of phosphate and this may carry over into solutions prepared in beakers and measuring cylinders. If most of your components appear to be contaminated with P_i, try switching to a phosphate-free detergent or segregate assay glassware from the normal laboratory wash.

8.2 How much enzyme should I use in my assay?

In a 200 µL reaction you should aim to add enough sufficient enzyme to generate 1-8 nmol of Pi (5- 40 µM). For any new enzyme it will be necessary to determine the extent of Pi production with serial dilutions of the enzyme. Plot the amount of Pi released versus amount of enzyme and select a dilution of enzyme that is in the linear range.

8.3 How much substrate should I use?

The amount of substrate hydrolyzed to Pi should not exceed 10-20% in an assay; otherwise the rate of Pi release with time may not be linear. However, the linear range for any given set of conditions can only be determined by experimentation and you will need to set up a time course.

To get a large assay window with only a modest % conversion of substrate, the initial concentration of the phosphorylated substrate in an assay will usually need to be 50-250 µM. If Pi production is between 10 µM and 40 µM the assay signal will normally be between 0.5 and 2.0 absorbance units (see Fig 1).

8.4 Should I subtract blanks from my assay samples and standard curve?

This comes down to personal preference but the main thing to consider is that for any single absorbance reading there are two or more components to that reading. This applies to all absorbance assays and is not specific to this assay. An appreciation of the different components is required to determine the best way of handling the controls and blanks, and whether to subtract blanks from the assay wells and standard curve before carrying out calculations.

For example, if we assume that the substrate is contaminated with free Pi, the single measured absorbance (Y1) for the assay wells is the sum of three separate components (i) the blank value due to the reagent alone, which is ~0.1 (ii) the signal due to contaminating Pi (iii) the signal due to Pi released from the substrate during the assay. The control wells (in this case wells with substrate but without enzyme) give a single absorbance reading (Y2) that is made up of two components, the blank value and the signal due to contaminating Pi in the substrate. Thus, subtraction of Y2 from Y1 subtracts the component due to contaminating Pi and also the blank component. The resulting value can therefore be used to calculate the amount of Pi formed using a *blank-subtracted* standard curve.

Whilst in the above example it was not necessary to subtract the measured blank value directly from the assay data (since subtraction of the control Y2 from Y1 achieved the same result) it is generally safer to subtract the blank value (i.e. water plus 0.25 volumes of mix and 0.1 volume of stabilizer) from the standards, assay wells and any control wells before calculations on the data are performed. In this way, regardless of how many controls need to be subtracted from the assay data you cannot inadvertently subtract the 'hidden' blank value more than once.

#Tip: you can do a single control that includes all assay components by using a different order of reagent addition. Add all components except the enzyme (but do not add water instead of enzyme) to triplicate wells, followed by the mix (ignore the fact that the enzyme is missing and add the usual 0.25 volumes of mix). Next, add the enzyme. (There are few, if any, enzymes that are active in the acidic medium). Five minutes later add the stabilizer and read the plates normally. This approach allows you to combine the enzyme and substrate in a single control well.

At what temperature should assays be carried out?

Enzyme assays are usually carried out in the range 20-37C. The preferred temperature will be determined to some extent by the lab equipment that is available. To compare data obtained on different days you should standardize the assay in respect of assay temperature. As far as the detection reagent is concerned the temperature of the initial enzyme assay is unimportant.

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