P_iColorLock™ Gold Phosphate Detection System

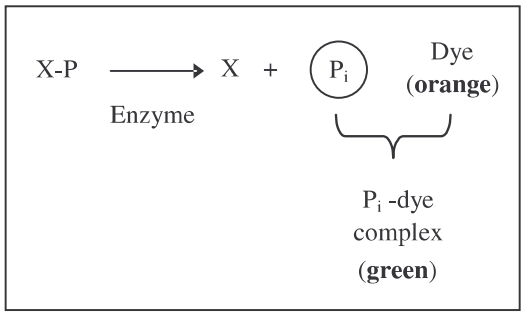
303-0030 **625/1560 Assays**
303-0124 **2500/6250 Assays**

1. INTRODUCTION

Phosphatases, ATPases and several other enzymes catalyse reactions in which inorganic phosphate (P_i) is released from an organic phosphorylated substrate. P_iColorLock™ Gold has been developed for measuring the activity of any P_i-generating enzyme in microplates. The reagent is specially formulated to give sensitive detection of P_i 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 P_iColorLock™ assay is based on the change in absorbance of the dye malachite green in the presence of phosphomolybdate complexes (see Fig 1). Unlike most malachite dye-based solutions, P_iColorLock Gold gives a stable end-point signal and is not prone to precipitation. Moreover, a special stabiliser ensures that the reagent can be used with acid labile substrates.

Fig 1. Principle of the P_iColorLock™ assay



2. INSTRUCTIONS

2.1 Overview of the P_iColorLock™ procedure

(i) Check for P_i contamination of enzyme sample and buffers.

(ii) Prepare a phosphate standard curve (P_i standard is provided) and set up enzyme assays.

(iii) Stop assays (this also initiates colour development).

(iv) After 5 min, add stabiliser*

(v) Read plates at a wavelength between 590nm and 650nm.

*Usually only required with acid labile substrates such as ATP.

2.2 Important considerations

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.

2.2.1 Preparation of 'Gold mix'

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

Important note: The Stabiliser is always added to the assay plate last, and it is always added on its own. Never add the stabiliser directly to the Gold mix. The volume of stabiliser needed is 0.1 vol. with respect to the initial assay volume (i.e. 10% of the initial assay volume is added).

2.2.2 Wavelength

The maximum signal for the P_iColorLock™ Gold reagent with P_i is obtained at ~635 nm but it is possible to achieve high sensitivity (>80% of the A_{635} 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.

2.2.3 Rate of color development

In most dye-based assays for P_i the plates are counted soon after the addition of the detection reagent, because of the high risk of precipitation of the P_i-dye complex or because of rising background signals. With P_iColorLock™ Gold, 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.

2.3. Preliminary checks for each new assay

In the discussion below, 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.

2.3.1 Checking for P_i contamination

Assay reagents and buffers that contain free P_i can give rise to an unacceptable assay background.

To check for contamination of reagents with free P_i prepare the solutions shown in Table 1. You will need concentrates of E, S & B, but thesolutions 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 P_i contamination and substrate stability.

Soln	Composition*
1	B + S (no enzyme)
2	B + E (no substrate)
3	B (no enzyme, no substrate)
4	P_i-free water

*Note: Where a component has been omitted the volume is made up with P_i-free water. 1ml 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 Gold mix (section 2.2.1). After 5 minutes (*it is important to wait 5 minutes*) add 0.1 volumes of stabiliser (i.e. 10% of the volume of the assay prior to the addition of Gold mix) and ensure thorough mixing. For example, if the assay volume is 200µl, add 50 µl of Gold mix and, five minutes later, 20 µl of the Stabiliser.

The volume of solutions 1 through 4 added to the wells need not be 200µl, as was given in the example above, but it should be the same as the volume of the enzyme-catalysed 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 (Section 2.2.2).

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) > Soln 3 (B alone), the stock substrate is almost certainly contaminated with free P_i.

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!).

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

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.

2.3.2. Acid stability of the substrate

If you wish to examine whether your substrate is unstable in acid, set up wells as described in section 2.3.1 and add water instead of the stabiliser. Acid-labile substrates will give a rising background signal as the levels of P_i increase through non-enzymatic release. Some phosphorylated substrates will degrade rapidly (e.g. ATP) in the absence of the stabiliser while others will degrade more slowly (e.g. ser/thr phosphopeptides). AMP (as used in nucleotidase assays) is completely stable in Gold mix.

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

2.3.3. Types of interference

The interference you are most likely to see is P_i 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.

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 P_iColorLock™ Gold 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	Precipitation
DMSO	2.5%	None
Detergents	0.03%	See footnote**

*The stated values refer to concentrations in the assay samples **before** the addition of ALS 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 ALS 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 catalysed reaction.

2.4. Standard curves

2.4.1 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 P_i needs to be accurately quantified (e.g. to calculate enzyme activity; Appendix II) a standard curve will be needed and contamination of assay components with free P_i will need to be checked (Section 2.3.1) and, if required, suitable corrections applied to the assay data.

2.4.2. Preparation of a standard curve

Prepare a set of P_i standards using the 0.1 mM P_i stock provided with the P_iColorLock™ Gold reagent, as indicated in Table 3. Set up triplicate wells of each standard.

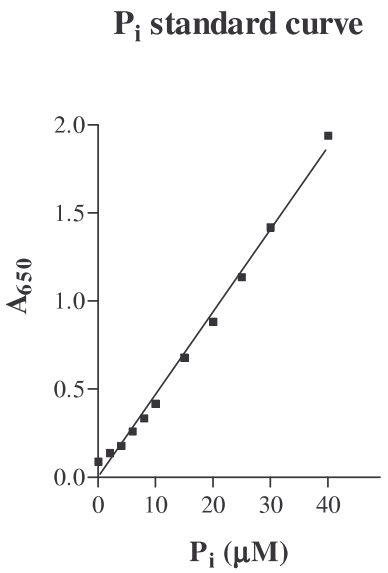
The volume of P_i standard added should be the same as the volume of the enzyme-catalysed reaction that you propose to run, so that the

depth of solution for both the standards and the assay samples is identical.

Add 0.25 volumes of Gold mix (section 2.2.1) to each well followed five minutes later by 0.1 volumes of stabiliser (i.e. 10% of the volume of the assay prior to addition of Gold mix). Allow sufficient time for the color to develop (Section 2.2.3) before counting plates in the range 590-660nm (Section 2.2.2).

Plot absorbance values versus concentration of P_i. Fig 2 below shows an example calibration curve that was obtained using a clear 96-well plate with 200 µl standard + 50µl Gold mix and 20 µl of stabiliser) read after 30 min at A_{650}.

Figure 2. Standard curve for P_iColorLock™ Gold



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 customise your standard curve if required.

2.5. Storage of reagent

The stabiliser should be stored at room temperature and the P_iColorLock™ Gold reagent and other kit components at 4°C. Under these storage conditions optimum performance will be observed for 12 months.

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

Table 3. Phosphate standards

Tube	0.1 mM P_i standard	Water	Concentration of P_i
	(µl)	(µl)	(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

3. ORDERING INFORMATION

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

#303-0030	30 ml	(625/1560 assays)
#303-0125	125ml	(2500/6250 assays)

Bulk quantities for HTS are always available: please contact us.

4. RELATED PRODUCTS AND SERVICES

4.1. Products

#303-0030 P_iColorLock™ ALS

P_iColorLock™ ALS is similar to P_iColorLock™ Gold but is added to samples in a volume ratio of 4:1 (c.f. ratio 1:4 for Gold reagent). P_iColorlock ALS is designed for assays in which P_i concentrations are in the range 25-175µM. Dye

complexes are extremely stable (for at least 16 hours). The ALS formulation is also compatible with acid labile substrates.

#501-0015 PiBind™ resin

PiBind™ resin has a high affinity for P_i and is used to remove contaminating P_i from buffer solutions and protein samples.

5. TROUBLE SHOOTING/FAQ

Q1. 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.

Q2. How much enzyme should I use in my assay?

Our free guide 'Enzyme units explained' which is available on our website will provide some help here. In a 200µl reaction you should aim to add enough sufficient enzyme to generate 1-8 nmol of P_i (5-40 µM). For any new enzyme it will be necessary to determine the extent of P_i production with serial dilutions of the enzyme. Plot the amount of P_i released versus amount of enzyme and select a dilution of enzyme that is in the linear range.

Q3. How much substrate should I use?

As a general rule, the amount of substrate hydrolysed to P_i should not exceed 10-20% in an assay; otherwise the rate of P_i 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 a Gold assay will usually need to be 50-250 µM. If P_i production is between 10 µM and 40µM the assay signal will normally be between 0.5 and 2.0 absorbance units (see Fig 2).

Q4. 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 actually two or more components to that reading. This applies to all absorbance assays, and is not specific to this particular assay. An appreciation of the different components is required in order 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 P_i, the single measured absorbance (Y_1) for the assay wells is the sum of *three* separate components (i) the blank value due to the Gold reagent alone, which is ~0.1 (ii) the signal due to contaminating P_i (iii) the signal due to P_i released from the substrate during the assay. The control wells (in this case wells with substrate but without enzyme) give a single absorbance reading (Y_2) that is made up of two components, the blank value and the signal due to contaminating P_i in the substrate. Thus subtraction of Y_2 from Y_1 subtracts the component due to contaminating P_i and also the blank component. The resulting value can therefore be used to calculate the amount of P_i 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 Y_2 from Y_1 achieved the same result) it is generally safer to subtract the blank value (i.e. water plus 0.25 volumes of Gold mix and 0.1 volume of stabiliser) 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 Gold mix (ignore the fact that the enzyme is missing and add the usual 0.25 volumes of Gold mix). Next, add the enzyme. (There are few, if any, enzymes that are active in the acidic Gold medium). Five minutes later add the stabiliser

and read the plates normally. This approach allows you to combine the enzyme and substrate in a single control well.

Q5. At what temperature should assays be carried out?

Enzyme assays are usually carried out in the range 20-37°C. 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 standardise the assay in respect of assay temperature. As far as the Gold detection reagent is concerned the temperature of the initial enzyme assay is unimportant.

Appendix II

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:

Step 1. Determine the *concentration* of P_i arising from the enzyme-catalysed reaction. This is determined from the measured absorbance value (after correction for any contaminating P_i; see FAQ 4) by reference to the standard curve. The value on the x-axis is a µM value (i.e. nmol P_i/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.

Step 2. Divide by the assay time in minutes (for the enzyme-catalysed reaction). This step gives *nmol of P_i 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).

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

Step 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 GOLD 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 GOLD mix), multiply the result from step 2 by 10.

Step 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 of 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.