

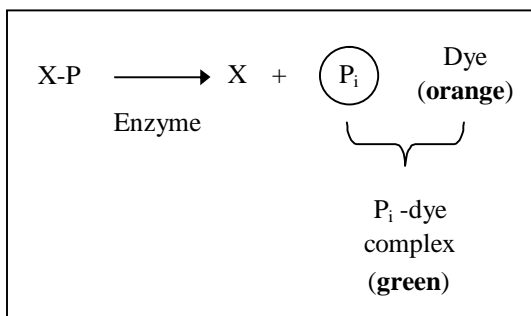


1. INTRODUCTION

Phosphatases, ATPases and several other enzymes catalyse reactions in which inorganic phosphate (P_i) is released from a substrate. P_iColorLock™ Gold has been developed for measuring the activity of any P_i-generating enzyme. The reagent is formulated to give sensitive detection of P_i. It provides an alternative to hazardous radioactive methods and other less sensitive colorimetric assays.

The measurement of P_i is based on the change in absorbance of malachite green in the presence of molybdate (Fig 1). Unlike other malachite dye formulations, P_iColorLock™ Gold gives a completely stable end-point signal and is not prone to precipitation. Moreover, a proprietary stabiliser ensures that P_iColorLock™ Gold can be used even with acid labile substrates.

Fig 1. Principle of the P_iColorLock™ assay



2. INSTRUCTIONS

2.1 Overview of the P_iColorLock™ procedure

- (i) Set up assays in microplates.
- (ii) Stop by addition of P_iColorLock™ Gold.
- (iii) Five minutes later, add stabiliser.*
- (iv) Read plates.

*Only required with acid labile substrates.

2.2 Assay set up

2.2.1 Preparation of 'GOLD mix'

Prepare 'Gold mix' shortly before the reagent is required by adding 1/100 vol. of Accelerator to P_iColorLock™ Gold reagent (e.g. for 10ml of reagent add 0.1ml of Accelerator).

2.2.2 Amount of 'Gold mix' per well

The Gold mix is added to P_i-containing samples in a volume ratio of 1:4 (i.e. the volume added is 25% of the initial assay volume). So, for a 100µl assay you would add 25µl of gold mix.

2.2.3 Amount of Stabiliser per well

The volume of stabiliser required is 10% of the initial assay volume (i.e. ignoring the volume of Gold mix added). So, for a 100µl assay you would add 10µl of Stabiliser.

Important note: The Stabiliser is always added to the assay plate last, and it is always added five minutes after the Gold mix. Never add the stabiliser directly to the Gold mix.

2.2.4 Time for color development

You should wait 30 minutes before reading the plate. However, the reading can be taken many hours later if required.

2.2.5 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 A₆₃₅ value) over a broad range of wavelengths (590-660 nm).

2.3. Preliminary checks for each new assay

2.3.1 Free phosphate

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

background. Before running assays, check that assays without enzyme give a low background signal (<0.1 OD units). If your enzyme contains phosphate buffer you will need to desalt or dialyse the enzyme prior to use.

2.3.2. Stability of the substrate

Without the Stabiliser, acid-labile substrates will give a rising background signal through non-enzymatic release of P_i . The stability of your substrate can easily be assessed by omitting the Stabiliser and counting the plate over a time course.

2.3.3. Compatibility with lab chemicals

Table 2 lists chemicals that are often used in enzyme assays, giving the expected type of interference (if any), and the acceptable range of concentrations (for reagents tested individually).

Table 2. Effects of lab chemicals in the P_i ColorLock™ 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 Gold mix.

**Very low concentrations of detergent are *more* likely to cause interference (precipitation) than high concentrations. If a detergent is required, use at least 0.03% concentration. Tween 20 is a preferred detergent. Triton X-100 is less well tolerated. SDS should not be used. Since the Gold mix is very acidic there is no need to add SDS to enzyme assays if the only purpose of this addition is to stop the enzyme catalysed reaction.

2.4. Standard curves

2.4.1 General considerations

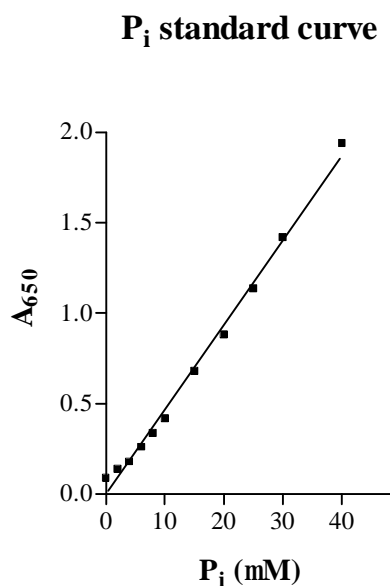
If *relative* absorbance values are more important than absolute values (e.g. drug screening applications), it is probably not necessary to set up a standard curve. However, if the amount of P_i needs to be accurately quantified to calculate enzyme activity a standard curve will be needed.

2.4.2. Preparation of a standard curve

Prepare a set of P_i standards using the 0.1 mM P_i stock provided, as indicated in Table 3. Set up triplicate wells of each dilution. The volume of P_i standard 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 Gold mix (section 2.2.1) to each well followed five minutes later by Stabiliser (section 2.2.3). Allow the color to develop (Section 2.2.4) before reading the plate (Section 2.2.5). Plot absorbance values versus concentration of P_i .

Fig 2 below shows an example calibration curve that was obtained using a 96-well plate with 200 μl standard + 50μl Gold mix and 20 μl of stabiliser) read after 30 min at A₆₅₀.

Figure 2. Standard curve for P_i ColorLock™ Gold



2.5. Storage of reagents

The stabiliser should be stored at room temperature and the PiColorLock™ Gold reagent and other kit components at 4°C. Under these conditions the reagents are stable for at least 12 months.

While the PiColorLock™ Gold reagent and Accelerator are stable separately for months, the Gold mix should NOT be stored for long periods. Only prepare quantities of Gold 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 |
|------|--------------------------------|-------|---------------------------------|
| | (ml) | (ml) | (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 |

3. Ordering information

#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 PiColorLock™ ALS

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

4.2. Assay services

A confidential rapid assay development service is available to clients who prefer to purchase PiColorLock™ reagent together with a protocol optimised for their particular substrate/enzyme. Contact customer service for further details.

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?

You should generally add sufficient enzyme to generate a signal between 0.5-2.0 absorbance units. 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 gives the required signal.

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. To get a reasonably 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 10-40µM the assay signal will be between 0.5 and 2.0 absorbance units (see Fig 2).

For other tips on setting up enzyme assays, including calculation enzyme activity, see our FAQ page and technical guides on our web site. Innovabiosciences.com.