

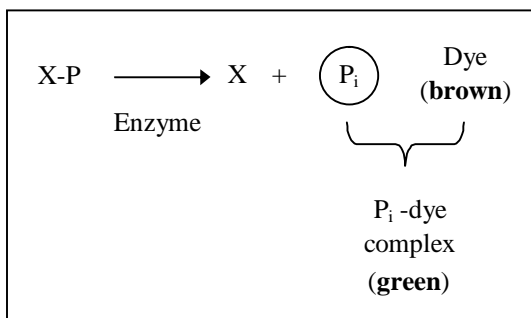


1. INTRODUCTION

Phosphatases, ATPases and several other enzymes catalyse reactions in which inorganic phosphate (P_i) is released from a substrate. P_iColorLock™ ALS 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™ ALS gives a completely stable end-point signal and is not prone to precipitation. Moreover, a proprietary stabiliser ensures that P_iColorLock™ ALS 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 or cuvettes.
- (ii) Stop by addition of P_iColorLock™ ALS.
- (iii) Five minutes later, add stabiliser.*
- (iv) Read plates or cuvettes.

*Only required with acid labile substrates.

2.2 Assay set up

2.2.1 Preparation of 'ALS mix'

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

2.2.2 Amount of 'ALS mix' per well

The ALS mix is added to P_i-containing samples in a volume ratio of 4:1 (i.e. the vol. of ALS mix added is 4 times the initial sample volume). So, for a 50µl sample you add 200µl of ALS mix.

2.2.3 Amount of Stabiliser per well

The volume of stabiliser required is 10% of the volume of ALS mix added. So, if you add 200 µl of ALS mix to your sample you add 20µl of Stabiliser five minutes later.

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

2.2.4 Time for color development

You should wait 30 minutes before reading the samples. However, the signal is extremely stable and readings can be taken hours later if required.

2.2.5 Wavelength

The maximum signal for the P_iColorLock™ ALS 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 some common assay components in the P_i ColorLock™ ALS assay

Component	Conc.*	Effect
Salts:		
NaCl	0-1 M	None
KCl	1 M	Slight signal loss
MgCl ₂	0-0.1 M	None
Thiols:		
DTT	0-0.1 mM	None/slight signal loss
DTT	0.1 mM	~5% signal loss
DTT	1 mM	~10% signal loss
βME	2 mM	None
Buffers:		
Tris	10-50 mM	None
Hepes	10-50 mM	None
Mes	10-50 mM	None
Mops	10-50 mM	None
Misc:		
DMSO	1-10% v/v	None
BSA	0.1 mg/ml	None
BSA	1 mg/ml	None
BSA	10 mg/ml	Slight increase in background, risk of precipitation
Glycerol	5%	None
Sucrose	5%	None
Detergents	0.05-0.2%	See footnote**

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

**Very low concentrations of detergent are *more* likely to cause interference (precipitation) than high concentrations. If a detergent is required, use >0.05%. Tween 20 is a preferred detergent. Triton X-100 is less well tolerated. SDS should not be used.

2.4. Standard curves

2.4.1 General considerations

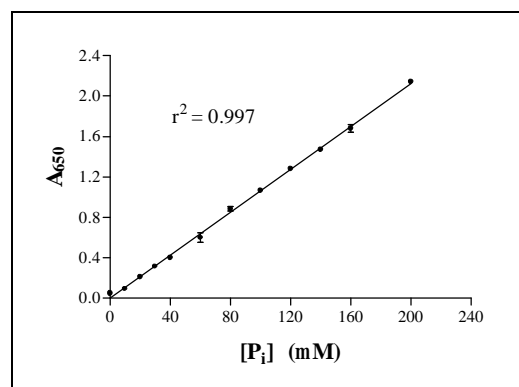
If *relative* absorbance values are more important than absolute values (e.g. drug screening applications), it is probably not necessary to set up a standard curve. However, if the amount of P_i needs to be accurately quantified to calculate enzyme activity a standard curve will be needed.

2.4.2. Preparation of a standard curve

Prepare a set of P_i standards using the 0.8 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 ALS 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).

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

Fig 2. Standard curve for P_i ColorLock™ ALS



2.5. Storage of reagents

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

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

Table 3. Phosphate standards

Tube	0.8 mM Pi standard	Water	Pi Conc.
	(ml)	(ml)	(mM)
1	100	300	200
2	80	320	160
3	70	330	140
4	60	340	120
5	50	350	100
6	40	360	80
7	30	370	60
8	20	380	40
9	15	385	30
10	10	390	20
11	5	395	10
12	0	400	0

3. Ordering information

#302-0125 125 ml (625/1560 assays)
#302-0500 500 ml (2500/6250 assays)

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

4. Related products and services

4.1. Products

#303-0030 PiColorLock™ Gold

PiColorLock™ Gold is similar to PiColorLock™ ALS but is added to samples in a volume ratio of 1:4 (c.f. ratio 4:1 for ALS reagent). PiColorlock Gold is designed for assays in which Pi concentrations are in the range 0-40µM. Like PiColorlock ALS, the Gold formulation is also compatible with acid labile substrates.

#501-0015 PiBind™ resin

PiBind™ resin has a high affinity for Pi and is used to remove contaminating Pi 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 Pi, 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 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 gives the required signal.

Q3. How much substrate should I use?

As a general rule, the amount of substrate hydrolysed to Pi should not exceed 10-20% in an assay; otherwise the rate of Pi 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 an 'ALS' assay will usually need to be 200-1000 µM. If Pi production is between 50-200µM 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 of enzyme activity, see our FAQ page and technical guides on our web site. <http://www.innovabiosciences.com>