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303-0030 Protocol

PiColorlock Gold Kit Protocol (303-0030)

1. INTRODUCTION
Phosphatases, ATPases and several other enzymes catalyse reactions in which inorganic phosphate (Pi) is released from a substrate. PiColorLock Gold has been developed for measuring the activity of any Pi-generating enzyme. The reagent is formulated to give sensitive detection of Pi . It provides an alternative to hazardous radioactive methods and other less sensitive colorimetric assays. The measurement of Pi is based on the change in absorbance of malachite green in the presence of molybdate (Example 1). Unlike other malachite dye formulations, Pi ColorLock Gold gives a completely stable end-point signal and is not prone to precipitation. Moreover, a proprietary stabiliser ensures that PiColorLock Gold can be used even with acid labile substrates.

Example 1. Principle of the PiColorLock assay
X-P --> (Enzyme) --> X + Pi and Dye (orange) = Pi-dye complex (green)

2. INSTRUCTIONS
2.1 Overview of the PiColorLock procedure
(i) Set up assays in microplates.
(ii) Stop by addition of PiColorLock 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 PiColorLock 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 Pi-containing samples in a volume ratio of 1:4 (i.e. the volume added is 25% of the initial assay volume). So, for a 100ul assay you would add 25ul 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 100ul assay you would add 10ul 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 PiColorLock Gold 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).

2.3. Preliminary checks for each new assay
2.3.1 Free phosphate
Assay reagents and buffers that contain free Pi 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 nonenzymatic release of Pi . 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
The examples below list 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).
Example 2. Effects of lab chemicals in the PiColorLock Gold assay

Salts:

NaCl --> 250mM concentration --> No Effect
KCl --> 250mM concentration --> No Effect
MgCl2 --> 25mM concentration --> No Effect
DTT --> 0.25mM concentration --> Slight signal loss
bME --> 0.5mM concentration --> No Effect
Tris --> 25mM concentration --> No Effect
Hepes --> 25mM concentration --> No Effect
Mes --> 25mM concentration --> No Effect
Mops --> 25mM concentration --> No Effect
BSA --> 0.1mg/ml concentration --> No Effect
BSA --> 1mg/mlconcentration --> Precipitation
DMSO --> 2.5% concentration --> 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 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 Pi 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 Pi standards using the 0.1mM Pi stock provided, as indicated in Example 3. Set up triplicate wells of each dilution. The volume of Pi 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 Pi.

2.5. Storage of reagents
The stabiliser should be stored at room temperature and the PiColorLock Gold reagent and other kit components at 4 degrees Celsius. 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.

Example 3. 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. Related 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 Pi concentrations are in the range 25-175uM. Like PiColorlock Gold, the ALS 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.

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 a Gold assay will usually need to be 50-250uM. If Pi production is between 10-40uM the assay signal will be between 0.5 and 2.0 absorbance units.

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 Pi, the single measured absorbance (Y1) 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 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.

While 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 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 20-37C. 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 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.