P_iPer™ Pyrophosphate Assay Kit

Table 1. Contents and storage information.

Material	Amount	Concentration	Storage	Stability
Amplex® Red reagent (Component A, MW = 257)	5 vials, 0.26 mg each	NA	• ≤–20°C • Desiccate • Protect from light	When stored as directed, kit components should remain stable for at least 6 months.
DMSO, anhydrous (Component B)	700 µL			
Maltose phosphorylase, recombinant form *Escherichia coli* (Component D)	250 U *			
Maltose, monohydrate (Component E, MW = 360.3)	20 mg			
Glucose oxidase from *Aspergillus niger* (Component F)	200 U †			
Horseradish peroxidase (HRP) (Component G)	50 U ‡			
Inorganic pyrophosphatase from baker's yeast (Component H)	2 U §			
Reaction buffer (Component C), 500 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.5	28 mL	5X		
Pyrophosphate standard (Component I), 50 mM sodium pyrophosphate	500 µL	NA		

***** 1 unit is defined as the amount of maltose phosphorylase that will convert maltose, in the presence of inorganic phosphate, to 1.0 µmole of D-glucose and D-glucose 1-phosphate per minute at pH 7.0 at 37°C. **†** 1 unit is defined as the amount of glucose oxidase that will oxidize 1.0 µmole of β-D-glucose to D-gluconolactone and H_2O_2 per minute at pH 5.1 at 35°C. **‡** 1 unit is defined as the amount of enzyme that will form 1.0 mg purpurogallin from pyrogallol in 20 seconds at pH 6.0 at 20°C. **§** 1 unit is defined as the amount of enzyme that will liberate 1.0 µmole of phosphate per minute at pH 7.2 at 25°C. NA = Not applicable.

Number of labelings: ~1,000 assays using either a fluorescence or an absorbance microplate reader and reaction volumes of 100 µL per assay.

Approximate fluorescence excitation/emission maxima: 563/587 nm

Introduction

Molecular Probes P_iPer™ Pyrophosphate Assay Kit provides an ultrasensitive assay that detects free pyrophosphate in solution through the formation of the fluorescent product resorufin. Because resorufin also has strong absorption, the assay can be performed either fluorometrically or spectrophotometrically. The kit can be used to detect pyrophosphate (PP_i) in a variety of samples or to monitor the kinetics of pyrophosphate release by a variety of enzymes, including: DNA and RNA polymerases, adenylate cyclase, and *S*-acetyl coenzyme A synthetase.

In the P_iPer™ Pyrophosphate Assay (Figure 1), inorganic pyrophosphatase hydrolyzes PP_i to two molecules of inorganic phosphate (P_i). The P_i then enters into the same cascade of reactions that is the basis for our P_iPer™ Phosphate Assay Kit (P22061). In the presence of P_i, maltose phosphorylase converts maltose to glucose 1-phosphate and glucose. Then glucose oxidase converts the glucose to gluconolactone and H_2O_2. Finally, with horseradish peroxidase (HRP) as a catalyst, the H_2O_2 reacts with the Amplex® Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) to generate resorufin, which has excitation and fluorescent emission maxima of approximately 563 nm and 587 nm, respectively (Figure 2).[1,2] The resulting increase in fluorescence or absorption is proportional to the amount of PP_i in the sample. By fluorescence, the kit can be used to detect as little as 0.8 µM PP_i (Figure 3A), or 3 µM by absorption (Figure 3B).

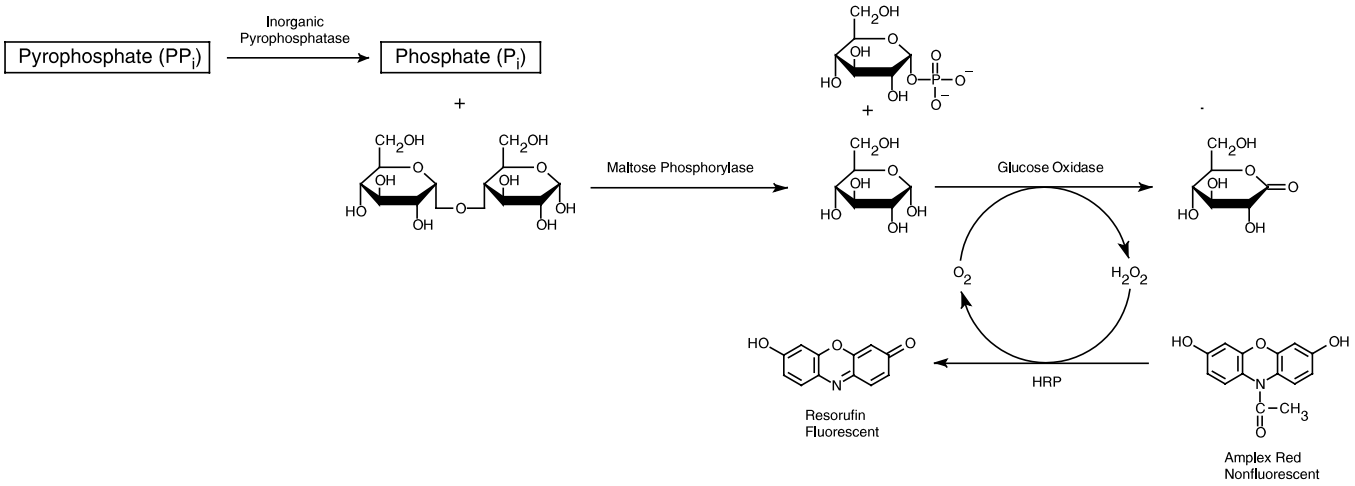

Figure 1. Principle of the P_iPer Pyrophosphate Assay. Inorganic pyrophosphatase converts pyrophosphate to two equivalents of inorganic phosphate. Next, in the presence of the inorganic phosphate, maltose phosphorylase converts maltose to glucose 1-phosphate and glucose. Then, glucose oxidase converts the glucose to gluconolactone and H_2O_2. Finally, with horseradish peroxidase (HRP) as a catalyst, the H_2O_2 reacts with the Amplex Red reagent to generate the highly fluorescent product, resorufin. The resulting increase in fluorescence or absorption is proportional to the amount of pyrophosphate in the sample.

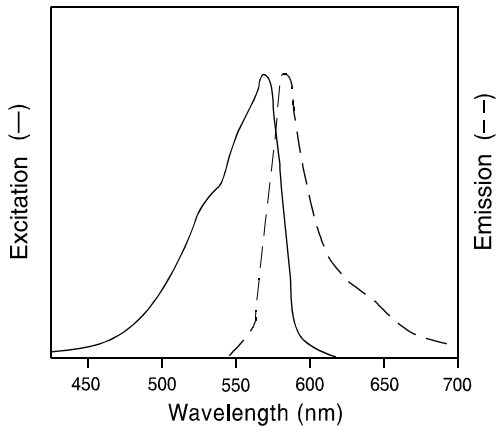

Figure 2. Normalized fluorescence excitation and emission spectra of resorufin, the product of the Amplex® Red reagent.

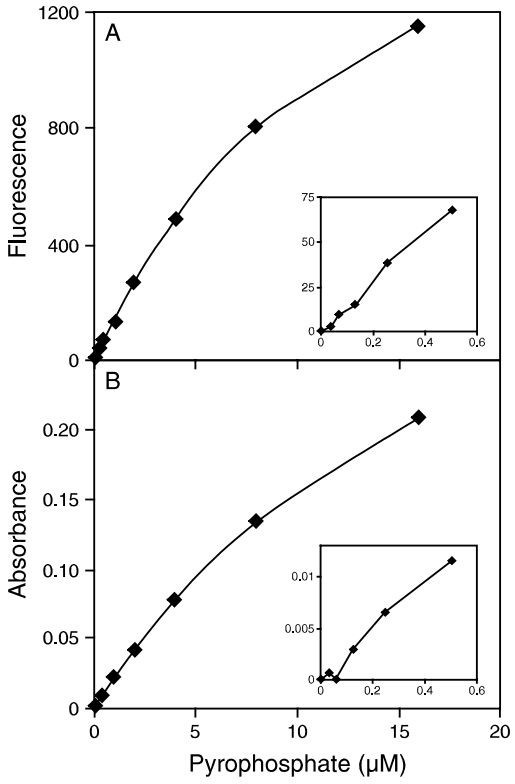

Figure 3. Detection of pyrophosphate using the P_iPer Pyrohosphate Assay Kit. Each reaction contained 50 µM Amplex® Red reagent, 0.01 U/mL inorganic pyrophosphatase, 2 U/mL maltose phosphorylase, 0.2 mM maltose, 1 U/mL glucose oxidase, and 0.2 U/mL HRP in 1X reaction buffer. Reactions were incubated at 37°C. After 60 minutes, fluorescence was measured in a fluorescence microplate reader using excitation at 530 ± 12.5 nm and fluorescence detection at 590 ± 17.5 nm (panel A) or absorbance at 576 ± 5 nm in an absorbance microplate reader. Data points represent the average of duplicate reactions. In panel A, a background value of 78 (arbitrary units) has been subtracted from each reading; in panel B, a background absorbance of 0.011 has been subtracted.

Allow the reagents to warm to room temperature before opening the vials. The Amplex® Red reagent is somewhat air sensitive. Once a vial of Amplex® Red reagent has been opened, the reagent should be used promptly.

Preparing the Stock Solutions for All Assay Protocols

1.1 Prepare a 10 mM stock solution of Amplex® Red reagent: Allow one vial of Amplex® Red reagent (Component A) and DMSO (Component B) to warm to room temperature. Just prior to use, dissolve the contents of the vial of Amplex® Red reagent (0.26 mg) in 100 µL DMSO. Each vial of Amplex® Red reagent is sufficient for approximately 200 assays, with a final reaction volume of 100 µL per assay. This stock solution should be stored frozen at ≤–20°C, protected from light.

1.2 Prepare a 1X working solution of reaction buffer by adding 5 mL of 5X reaction buffer stock solution (Component C) to 20 mL of deionized water (dH_2O). This 25 mL volume of 1X reaction buffer is sufficient for approximately 200 assays of 100 µL each, with a 5 mL excess for making stock solutions and dilutions.

Note: The absorption and fluorescence of resorufin are pH-dependent. Below the pK_a (~6.0), the absorption maximum shifts to ~480 nm and the fluorescence quantum yield is markedly lower. In addition, the Amplex® Red reagent is unstable at high pH (>8.5). For these reasons, the reaction should be performed at pH 7–8, for example by using the provided reaction buffer (pH 7.5).

1.3 Prepare a 2 U/mL stock solution of inorganic phosphatase by dissolving the contents of the vial of inorganic phosphatase (Component H) in 1.0 mL of 1X reaction buffer. After use, the remaining solution should be divided into small aliquots and stored frozen at ≤–20°C.

1.4 Prepare a 200 U/mL stock solution of maltose phosphorylase by dissolving the contents of the vial of maltose phosphorylase (Component D) in 1.25 mL of 1X reaction buffer. After use, the remaining solution should be divided into small aliquots and stored frozen at ≤–20°C.

1.5 Prepare a 40 mM stock solution of maltose by dissolving the contents of the vial of maltose (Component E) in 1.39 mL of 1X reaction buffer. After use, the remaining solution should be stored frozen at ≤–20°C.

1.6 Prepare a 200 U/mL solution of glucose oxidase by dissolving the contents of the vial of glucose oxidase (Component F) in 1.0 mL of 1X reaction buffer. After use, the remaining solution should be divided into small aliquots and stored frozen at ≤–20°C.

1.7 Prepare a 100 U/mL stock solution of horseradish peroxidase (HRP) by dissolving the contents of the vial of HRP (Component G) in 500 µL of 1X reaction buffer. After use, the remaining solution should be divided into small aliquots and stored frozen at ≤–20°C.

1.8 If desired, prepare a 20 mM H_2O_2 working solution by diluting a stock solution of H_2O_2 (not provided) with dH_2O. For instance, a 20 mM H_2O_2 working solution can be prepared from a 3% H_2O_2 stock solution by diluting 23 µL of 3% H_2O_2 into 977 µL of dH_2O.

Note: 3% H_2O_2 stock solutions are usually stabilized to slow degradation; the 20 mM H_2O_2 working solution will be less stable and should be used promptly.

Experimental Protocols

Three assay protocols are described below. The section *Stock Solution Preparation* applies to all protocols. The basic assay protocols are described in the sections: *Assaying for Pyrophosphate* and *Assaying for Enzyme Activity*. In addition, there is a protocol describing the use of rigorous controls, for enzyme activity assays where user-supplied reagents may introduce contaminants (e.g., P_i, PP_i or glucose) that would interfere with the data interpretation if not fully accounted for. This section is: *Managing Background in Enzyme-Activity Assays*. Before proceeding with the applicable protocol, please read it through completely.

The assay protocols are designed for detection by means of either a fluorescence or absorbance multiwell plate reader. For detection with a standard fluorometer or spectrophotometer, the volumes must be increased accordingly.

Assaying for Pyrophosphate

The following protocol describes the basic assay for PP_i in a total volume of 100 µL per microplate well. The volumes recommended here are sufficient for ~100 assays.

Thiol Contamination

The product of the Amplex® Red reaction, resorufin, is unstable in the presence of thiols such as dithiothreitol (DTT) or 2-mercaptoethanol. For this reason, the final DTT or 2-mercaptoethanol concentration in the reaction should be less than 10 µM.

Phosphate contamination

The first step in the P_iPer™ Pyrophosphate Assay scheme (see Figure 1) is the conversion of PP_i to P_i. The P_i then enters a cascade of reactons that ultimately generates resorufin, which is detected by either fluorescence or absorbance. Thus, the pyrophosphate assay is also an extremely sensitive assay for inorganic phosphate. The reagents provided with the kit are certified to be low in contaminating phosphate; however, phosphate contamination can arise from impure water, traces of phosphate buffer in samples to be assayed, or from dirty glassware. In many instances, by the rigorous use of controls, some phosphate contamination can be tolerated and can be subtracted out in the analysis of the data.

Glucose contamination

The mechanism of the P_iPer™ Pyrophosphate Assay (see Figure 1) involves first the generation of P_i from PP_i by the action of inorganic pyrophosphatase, next glucose is generated from maltose and P_i by the action of maltose phosphorylase and then glucose is converted to gluconolactone and H_2O_2 by the action of glucose oxidase. Thus, glucose contamination of reagents is potentially a problem. Likewise, contamination by enzymes, such as α-glucosidase maltase, which can digest maltose to glucose, could cause aberrant measurements. The reagents provided in the kit are certified to be low in contaminating glucose and α-glucosidase. As with phosphate contamination, some glucose contamination can be tolerated and accounted for by the use of appropriate controls.

2.1 Prepare a PP_i standard curve: Dilute the appropriate amount of the 50 mM pyrophosphate standard solution (Component I) into 1X reaction buffer (prepared in step 1.2) to produce PP_i concentrations of 0 to 50 µM. Use 1X reaction buffer without PP_i as a negative control. A volume of 50 µL will be used for each reaction. Please note that the PP_i concentrations will be twofold lower in the final reaction volume.

2.2 Dilute the experimental samples in 1X reaction buffer. A volume of 50 µL will be used for each reaction.

2.3 If desired, prepare a positive control by diluting the 20 mM H_2O_2 working solution (prepared in step 1.8) to 10 µM in 1X reaction buffer.

2.4 Pipet 50 µL of the diluted samples and controls into separate wells of a microplate.

2.5 Prepare a working solution of 100 µM Amplex® Red reagent containing 0.02 U/mL inorganic pyrophosphatase, 4 U/mL maltose phosphorylase, 0.4 mM maltose, 2 U/mL glucose oxidase, and 0.4 U/mL HRP by adding:

- 4.68 mL of 1X reaction buffer.
- 50 µL of the Amplex® Red reagent stock solution (prepared in step 1.1)
- 50 µL of the inorganic pyrophosphatase stock solution (prepared in step 1.3)
- 100 µL of the maltose phosphorylase stock solution (prepared in step 1.4)
- 50 µL of the glucose oxidase (prepared in step 1.6)
- 20 µL of the HRP stock solution (prepared in step 1.7)
- 50 µL of the maltose stock solution (prepared in step 1.5; add last)

This 5 mL volume is sufficient for ~100 assays. Prepare only the amount needed for the experiment at hand. USE SOON AFTER PREPARATION, e.g. within 15 minutes. Note that the final concentrations of each component will be twofold lower in the final reaction volume.

2.6 Begin the reactions by adding 50 µL of the Amplex® Red reagent/inorganic pyrophosphatase/maltose phosphorylase/maltose/glucose oxidase/HRP working solution to each microplate well containing the samples and controls.

2.7 Incubate the reactions for 30 minutes or longer at 37°C, protected from light. Because the assay is continuous (not terminated), fluorescence, or absorbance, may be measured at multiple time points to follow the kinetics of the reactions.

2.8 Resorufin, the Amplex® Red reagent reaction product, can be detected either fluorometrically or spectrophotometrically. For fluorescence detection, use a fluorescence microplate reader set for excitation in the range of 530–560 nm and emission detection at ~590 nm (see Figure 2). For absorbance detection, use a microplate reader set for absorbance at ~565 nm.

2.9 For each point, correct for background fluorescence, or absorbance, by subtracting the values derived from the no-pyrophosphate control.

Assaying for Enzyme Activity

The following protocol provides a guideline for using the P_iPer™ Pyrophosphate Assay Kit to measure the activity of a pyrophosphate-generating enzyme. The enzyme, and the specific substrate for the enzyme, must be supplied by the user. The volumes recommended here are sufficient for ~100 assays, each containing a volume of 100 µL. In cases where either the enzyme or the substrate is possibly contaminated with P_i, PP_i or glucose, see *Managing Background in Enzyme-Activity Assays*, which follows the basic assay.

3.1 Dilute the samples containing the pyrophosphate-generating enzyme in 1X reaction buffer (prepared in step 1.2). A volume of 50 µL will be used for each reaction.

3.2 Set aside a sample of 1X reaction buffer without enzyme as a negative control. A volume of 50 µL will be used.

3.3 If desired, prepare a positive control by diluting the 20 mM H_2O_2 working solution (prepared in step 1.8) to 10 µM in 1X reaction buffer.

3.4 Pipet 50 µL of the diluted samples and controls into separate wells of a microplate.

3.5 Prepare a working solution of 100 µM Amplex® Red reagent containing 0.02 U/mL inorganic pyrophosphatase, 4 U/mL maltose phosphorylase, 0.4 mM maltose, 2 U/mL glucose oxidase, 0.4 U/mL HRP, and the substrate of the pyrophosphate-generating enzyme by adding:

- 4.68 mL of 1X reaction buffer (minus x µL, the substrate volume)
- 50 µL of the Amplex® Red reagent stock solution (prepared in step 1.1)
- 50 µL of the inorganic pyrophosphatase stock solution (prepared in step 1.3)
- 100 µL of the maltose phosphorylase stock solution (prepared in step 1.4)
- 50 µL of the glucose oxidase (prepared in step 1.6)
- 20 µL of the HRP stock solution (prepared in step 1.7)
- x µL of the substrate stock solution, as appropriate
- 50 µL of the maltose stock solution (prepared in step 1.5; add last)

This 5 mL volume is sufficient for ~100 assays. Prepare only the amount needed for the experiment at hand. USE SOON AFTER PREPARATION, e.g. within 15 minutes. Note that the final concentrations of each component will be twofold lower in the final reaction volume.

3.6 Begin the reactions by adding 50 µL of the Amplex® Red reagent/inorganic pyrophosphatase/maltose phosphorylase/maltose/glucose oxidase/HRP/substrate working solution to each microplate well containing the samples and controls.

3.7 Incubate the reactions for 30 minutes or longer at 37°C, protected from light. Because the assay is continuous (not terminated), fluorescence, or absorbance, may be measured at multiple time points to follow the kinetics of the reactions.

3.8 Resorufin, the Amplex® Red reagent reaction product, can be detected either fluorometrically or spectrophotometrically. For fluorescence detection, use a fluorescence microplate reader set for excitation in the range of 530–560 nm and emission detection at ~590 nm (see Figure 2). For absorbance detection, use a microplate reader set for absorbance at ~565 nm.

3.9 For each point, correct for background fluorescence by subtracting the values derived from the no-enzyme control.

Managing Background in Enzyme-Activity Assays

The following, a modification of the basic enzyme-activity assay, is recommended in situations where there is possible P_i, PP_i, or glucose contamination of the enzyme to be assayed, for example when impure extracts are used. The protocol incorporates a full set of controls for assessment and management of background activity. The method also controls for possible P_i, PP_i, or glucose contamination of the enzyme substrate. As described, the volumes are sufficient for ~100 assays and full set of controls, each reaction in a 100 µL volume.

4.1 Perform steps 3.1–3.4 exactly as above, except prepare sufficient volumes to have two 50 µL volumes of each sample and controls, and load two sets of microplate wells.

4.2 Prepare the working solution, including the enzyme substrate, exactly as in step 3.5.

4.3 Prepare a working solution, exactly as in step 3.5, except omit the enzyme substrate.

4.4 Begin one set of reactions, as in step 3.6, by adding 50 µL of the full Amplex® Red working solution (Amplex® Red reagent/inorganic pyrophosphatase/maltose phosphorylase/maltose/glucose oxidase/HRP/substrate working solution) to each microplate well containing the samples and controls. These are the **Full Reactions** (containing both enzyme and substrate) and one **No-Enzyme Control Reaction** (containing substrate, but no enzyme).

4.5 Begin another set of reactions by adding 50 µL of the no-substrate Amplex® Red working solution (Amplex® Red reagent/inorganic pyrophosphatase/maltose phosphorylase/maltose/glucose oxidase/HRP working solution) to each microplate well containing the second set of samples and controls. These are the **No-Substrate Control Reactions** (containing variable amounts of enzyme, but no substrate) and one **No-Enzyme/No-Substrate Control Reaction**.

4.6 Incubate the reactions and measure the fluorescence, or absorbance, exactly as in steps 3.7 and 3.8.

4.7 Analyze the data using the following logic:

Let **A** = the value for the **Full Reaction**. This value represents the enzymatic activity of the reaction plus backgrounds: the enzyme background (e.g., P_i, PP_i, or glucose contamination of the enzyme); the substrate background (e.g., P_i, PP_i, or glucose contamination of the substrate); and the reagent background (e.g., trace P_i, PP_i, or glucose contamination of the kit's reagents plus background fluorescence, or absorption, of the Amplex® Red reagent and spontaneous conversion of the Amplex® Red reagent to resorufin).

Let **B** = the value for the **No-Enzyme Control Reaction**. This value represents the substrate background plus the reagent background.

Let **C** = the value for the **No-Substrate Control Reaction**. This value represents the enzyme background plus the reagent background. Note that, for every Full Reaction, there should be a corresponding No-Substrate Reaction.

Let **D** = the value for the **No-Enzyme/No-Substrate Control Reaction**. This value represents the reagent background.

Thus, the fluorescence, or absorbance, value corresponding to enzyme activity alone is calculated as follows: **Enzyme Activity = A – B – C + D**. (Note that D is *added* in the equation.)

References

1. Anal Biochem 253, 162 (1997); **2.** J Immunol Methods 202, 133 (1997).

Product List **Current prices may be obtained from our website or from our Customer Service Department.**

Cat #	Product Name	Unit Size
P22062	P_iPer™ Pyrophosphate Assay Kit *1000 assays*	1 kit

Contact Information

Molecular Probes, Inc.
29851 Willow Creek Road
Eugene, OR 97402
Phone: (541) 465-8300
Fax: (541) 335-0504

Customer Service:
6:00 am to 4:30 pm (Pacific Time)
Phone: (541) 335-0338
Fax: (541) 335-0305
probesorder@invitrogen.com

Toll-Free Ordering for USA:
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Order Fax: (800) 438-0228

Technical Service:
8:00 am to 4:00 pm (Pacific Time)
Phone: (541) 335-0353
Toll-Free (800) 438-2209
Fax: (541) 335-0238
probestech@invitrogen.com

Invitrogen European Headquarters
Invitrogen, Ltd.
3 Fountain Drive
Inchinnan Business Park
Paisley PA4 9RF, UK
Phone: +44 (0) 141 814 6100
Fax: +44 (0) 141 814 6260
Email: euroinfo@invitrogen.com
Technical Services: eurotech@invitrogen.com

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