Revised: 01–September–2004

P_iPer™ Phosphate Assay Kit (P22061)

Quick Facts

Storage upon receipt:
- ≤–20°C
- Desiccate
- Protect from light

Abs/Em: 563/587 nm

Introduction

Molecular Probes' P_iPer™ Phosphate Assay Kit provides an ultrasensitive assay that detects free phosphate 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 inorganic phosphate (P_i) in a variety of samples or to monitor phosphate release by a variety of enzymes, including: ATPases, GTPases, 5′-nucleotidase, protein phosphatases, acid and alkaline phosphatases and phosphorylase kinase. Furthermore, the assay can be modified to detect virtually any naturally occurring organic phosphate molecule by including an enzyme that can specifically digest the organic phosphate to liberate inorganic phosphate.

In the P_iPer Phosphate Assay (Figure 1), maltose phosphorylase converts maltose (in the presence of P_i) 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 resor-ufin, which has absorption 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 P_i in the sample. By fluorescence, the kit can be used to detect as little as 800 nM P_i (Figure 3).

Materials

Kit Contents

- **Amplex Red reagent** (MW = 257, Component A), five vials, each containing 0.26 mg
- **Dimethylsulfoxide (DMSO), anhydrous** (Component B), 700 µL
- **5X Reaction Buffer** (Component C), 28 mL of 0.5 M Tris-HCl, pH 7.5
- **Maltose phosphorylase, recombinant from *Escherichia coli*** (Component D), 250 U, where 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

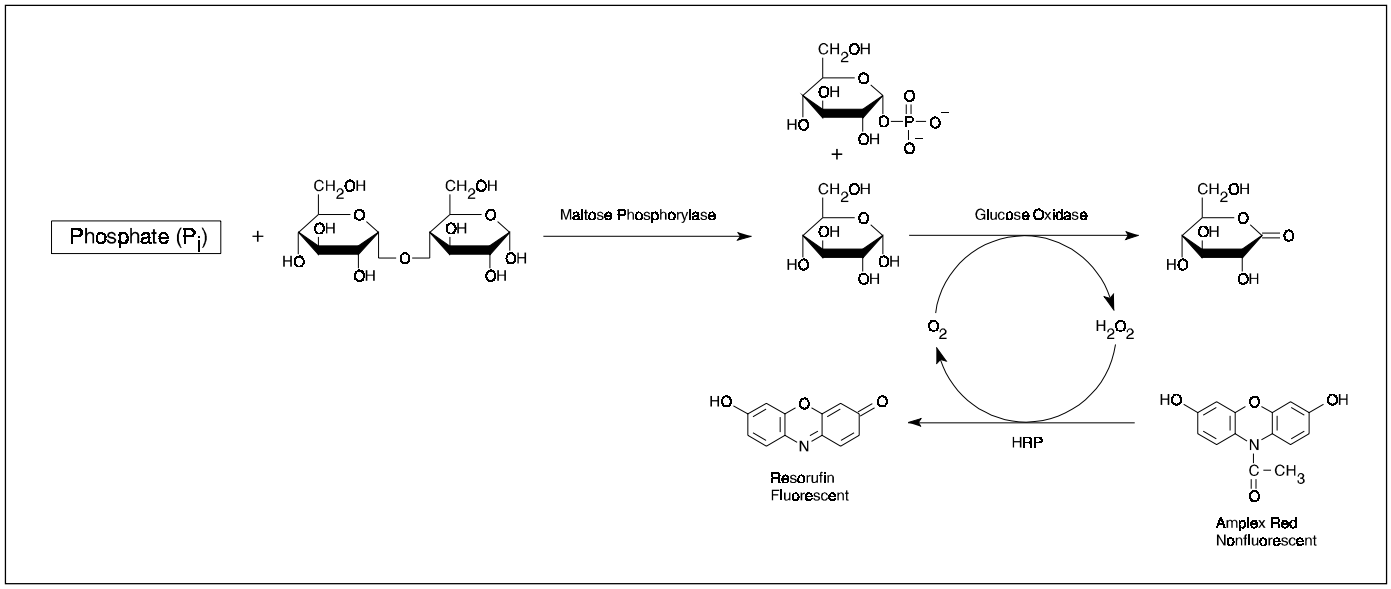

Figure 1. *Principle of the P_iPer Phosphate Assay. In the presence of inorganic phosphate, maltose phosphorylase converts maltose to glucose 1-phosphate and glucose. Then, glucose oxidase converts the glucose to gluconolactone and H_2O_2. Finally, with horseradish peroxidase (HRP) as a catalyst, the 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 P_i in the sample.*

- **Maltose, monohydrate** (MW = 360.3, Component E), 20 mg
- **Glucose oxidase from *Aspergillus niger*** (Component F), 200 U, where 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
- **Horseradish peroxidase (HRP)** (Component G), 50 U, where 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
- **Phosphate standard** (Component H), 500 µL of 50 mM potassium phosphate
- **Hydrogen peroxide (H_2O_2)** (MW = 34, Component I), 500 µL of a stabilized ~3% solution; the actual concentration is indicated on the component label

Each kit provides sufficient reagents for approximately 1000 assays using either a fluorescence or an absorbance microplate reader and reaction volumes of 100 µL per assay.

Storage and Handling

Upon receipt, the kit should be stored at ≤–20°C, protected from light. Stored properly, the kit's components should remain stable for at least six months. 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. PROTECT THE AMPLEX RED REAGENT FROM LIGHT.

Experimental Protocols

Several protocols are described below. The section *Stock Solution Preparation* applies to all of the assay protocols. The basic assay protocols are described in the sections: *Assay for Inorganic Phosphate*, *Assay for Enzyme Activity* and *Assay for Organic Phosphate*. In addition, there are two protocols describing the use of rigorous controls, for situations where user-supplied reagents may introduce contaminants (e.g. P_i or glucose) that would interfere with the data interpretation if not fully accounted for. These sections are: *Background Management in Enzyme-Activity Assays* and *Background Management in Organic-Phosphate Assays*. Before proceeding with the applicable protocol, please read it through, in its entirety. Please also read the *Protocol Notes* section, at the end.

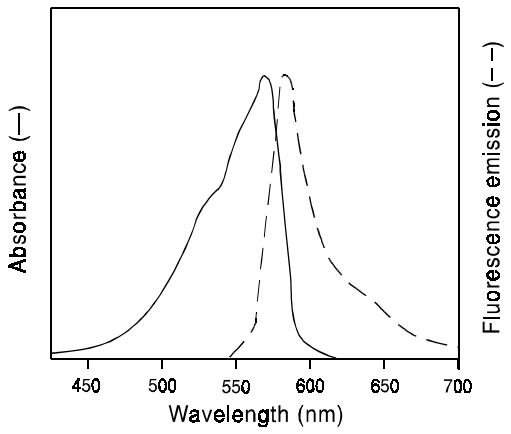

Figure 2. *Normalized absorption and fluorescence emission spectra of resorufin, the product of the Amplex Red reagent.*

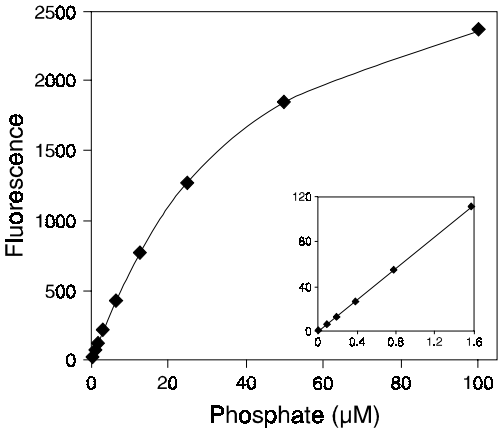

Figure 3. *Detection of inorganic phosphate using the P_iPer Phosphate Assay Kit. Each reaction contained 50 µM Amplex Red reagent containing 2 U/mL maltose phosphorylase, 1 mM maltose, 1 U/mL glucose oxidase and 0.2 U/mL HRP in 1X Reaction Buffer. Reactions were incubated at 37ºC. After 60 minutes, fluorescence was measured in a fluorescence microplate reader using excitation at 530 ± 12.5 nm and fluorescence detection at 590 ± 17.5 nm. Data points represent the average of duplicate reactions, and a background value of 43 (arbitrary units) has been subtracted from each.*

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.

Stock Solution Preparation — 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.

1.3 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.

1.4 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.

1.5 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.

1.6 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.

1.7 Prepare a 20 mM H_2O_2 working solution by diluting the ~3% H_2O_2 stock solution (Component I) into the appropriate volume of dH_2O. The actual H_2O_2 concentration is indicated on the component label. For instance, a 20 mM H_2O_2 working solution can be prepared from a 3.0% H_2O_2 stock solution by diluting 23 µL of 3.0% H_2O_2 into 977 µL of dH_2O. Please note that although the ~3% H_2O_2 stock solution has been stabilized to slow degradation, the 20 mM H_2O_2 working solution will be less stable and should be used promptly.

Assay for Inorganic Phosphate

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

2.1 Prepare a P_i standard curve: Dilute the appropriate amount of the 50 mM phosphate standard solution (Component H) into 1X Reaction Buffer (prepared in step 1.2) to produce P_i concentrations of 0 to 100 µM. Use 1X Reaction Buffer without P_i as a negative control. A volume of 50 µL will be used for each reaction. Please note that the P_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 1.7) 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 4 U/mL maltose phosphorylase, 0.4 mM maltose, 2 U/mL glucose oxidase and 0.4 U/mL HRP by adding:

- 4.73 mL of 1X Reaction Buffer.
- 50 µL of the Amplex Red reagent stock solution (prepared in step 1.1)
- 100 µL of the maltose phosphorylase stock solution (prepared in step 1.3)
- 50 µL of the glucose oxidase (prepared in step 1.5)
- 20 µL of the HRP stock solution (prepared in step 1.6)
- 50 µL of the maltose stock solution (prepared in step 1.4; 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/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-phosphate control.

Assay for Enzyme Activity

The following protocol provides a guideline for using the P_iPer Phosphate Assay Kit to measure the activity of a phosphate-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 or glucose, see *Background Management in Enzyme-Activity Assays*, which follows the basic assay.

3.1 Dilute the samples containing the phosphate-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 1.7) 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 4 U/mL maltose phosphorylase, 0.4 mM maltose, 2 U/mL glucose oxidase, 0.4 U/mL HRP and the organic phosphate substrate of the phosphate-generating enzyme by adding:

- 4.73 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)
- 100 µL of the maltose phosphorylase stock solution (prepared in step 1.3)
- 50 µL of the glucose oxidase (prepared in step 1.5)
- 20 µL of the HRP stock solution (prepared in step 1.6)
- x µL of the substrate stock solution, as appropriate
- 50 µL of the maltose stock solution (prepared in step 1.4; 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/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.

Background Management in Enzyme-Activity Assays

The following, a modification of the basic enzyme-activity assay, is recommended in situations where there is possible phosphate (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 phosphate (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/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/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. phosphate or glucose contamination of the enzyme); the substrate background (e.g. phosphate or glucose contamination of the substrate); and the reagent background (e.g. trace phosphate 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 Control 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.)

Assay for Organic Phosphate

The following protocol provides a guideline for using the P_iPer Phosphate Assay Kit as an assay for virtually any naturally occurring organic phosphate (e.g. ATP, GTP, creatine phosphate, acetyl phosphate, etc.). The assay requires the use of an enzyme (user-supplied) that can specifically digest the organic phosphate to inorganic phosphate. The inorganic phosphate is then detected by the standard P_iPer reaction. As described, the volumes are sufficient for ~100 assays, each in a 100 µL volume. In cases where either the enzyme or the substrate is possibly contaminated with P_i or glucose, see *Background Management in Organic-Phosphate Assays*, which follows the basic assay.

5.1 Prepare an organic-phosphate standard curve: Dilute a stock solution of the organic phosphate using 1X Reaction Buffer (prepared in step 1.2) to produce organic-phosphate concentrations of 0 to 100 µM. Use 1X Reaction Buffer without phosphate as a negative control. A volume of 50 µL will be used for each reaction. Please note that the organic-phosphate concentrations will be twofold lower in the final reaction volume.

5.2 Dilute the experimental samples containing the organic phosphate in 1X Reaction Buffer (prepared in step 1.2). A volume of 50 µL will be used for each reaction.

5.3 If desired, prepare a positive control by diluting the 20 mM H_2O_2 working solution (prepared in step 1.7) to 10 µM in 1X Reaction Buffer.

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

5.5 Prepare a working solution of 100 µM Amplex Red reagent containing 4 U/mL maltose phosphorylase, 0.4 mM maltose, 2 U/mL glucose oxidase, 0.4 U/mL HRP and the enzyme specific for the organic phosphate by adding:

- 4.73 mL of 1X Reaction Buffer (minus x µL, the enzyme volume)
- 50 µL of the Amplex Red reagent stock solution (prepared in step 1.1)

- 100 µL of the maltose phosphorylase stock solution (prepared in step 1.3)
- 50 µL of the glucose oxidase stock solution (prepared in 1.5)
- 20 µL of the HRP stock solution (prepared in 1.6)
- x µL of the enzyme stock solution, as appropriate
- 50 µL of the maltose stock solution (prepared in 1.4; 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.

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

5.7 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.

5.8 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.

5.9 For each point, correct for background fluorescence, or absorbance, by subtracting the values derived from the no-organic phosphate control. The values thus determined can be converted to meaningful units by comparison to a standard curve, prepared from the values obtained from the standard-curve reactions.

Background Management for Organic-Phosphate Assays

The following, a modification of the basic organic-phosphate assay, is recommended in situations where there is possible inorganic phosphate (or glucose) contamination of the organic phosphate samples. The protocol incorporates a full set of controls for the assessment and management of background activity. The method also controls for possible phosphate (or glucose) contamination of the enzyme used for the digestion of the organic phosphate. As described, the volumes are sufficient for ~100 assays and a full set of controls, each reaction in a 100 µL volume.

6.1 Perform steps 5.1–5.4 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.

6.2 Prepare a working solution, including the enzyme specific for the organic phosphate, exactly as in step 5.5.

6.3 Prepare a working solution, exactly in step 5.5, except omit the enzyme specific for the organic phosphate.

6.4 Begin one set of reactions, as in step 5.6, by adding 50 µL of the full Amplex Red working solution (Amplex Red reagent/ maltose phosphorylase/maltose/glucose oxidase/HRP/enzyme working solution) to each microplate well containing the samples and controls. These are the **Full Reactions** (containing both organic phosphate and enzyme) and one **No-Organic–Phosphate Control Reaction** (containing enzyme, but no organic phosphate substrate).

6.5 Begin another set of reactions by adding 50 µL of the no-enzyme Amplex Red working solution (Amplex Red reagent/ maltose phosphorylase/maltose/glucose oxidase/HRP working solution) to each well containing the second set of samples and controls. These are the **No-Enzyme Control Reactions** and one **No-Organic–Phosphate/No-Enzyme Control Reaction**.

6.6 Incubate the reactions and measure the fluorescence, or absorbance, as in steps 5.7 and 5.8.

6.7 Analyze the data using the following logic:

Let **A** = the value for the **Full Reaction**. This value represents the fluorescence, or absorbance, due to the detection of organic phosphate plus that due to backgrounds: the organic-phosphate background (e.g. phosphate or glucose contamination of the organic phosphate); the enzyme background (e.g. phosphate or glucose contamination of the enzyme specific for the organic phosphate); and the reagent background (e.g. trace phosphate 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-Organic–Phosphate Control Reaction**. This value represents the enzyme background plus the reagent background.

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

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

Thus, the fluorescence, or absorbance, value corresponding to detection of organic phosphate alone is calculated as follows: **Organic Phosphate = A - B - C + D**. (Note that D is *added* in the equation.) The values thus determined can be converted to meaningful units by comparison to a standard curve, prepared from the values (corrected analogously) obtained from the standard-curve reactions.

Protocol Notes

- **Reaction buffer pH:** 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).

- **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 P_iPer Phosphate Assay Kit provides 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 Phosphate Assay (see Figure 1), involves the generation of glucose from maltose and P_i by the action of maltose phosphorylase and the subsequent conversion of glucose 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.

References

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

Product List

Current prices may be obtained from our Web site or from our Customer Service Department.

Cat #	Product Name	Unit Size
P22061	P_iPer™ Phosphate Assay Kit *1000 assays*	1 kit

Contact Information

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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 ● order@probes.com

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Technical Assistance: 8:00 am to 4:00 pm (Pacific Time)
Phone: (541) 335-0353 ● Toll-Free: (800) 438-2209
Fax: (541) 335-0238 ● tech@probes.com

Molecular Probes Europe BV
Poortgebouw, Rijnsburgerweg 10
2333 AA Leiden, The Netherlands
Phone: +31-71-5233378 ● Fax: +31-71-5233419

Customer Service: 9:00 to 16:30 (Central European Time)
Phone: +31-71-5236850 ● Fax: +31-71-5233419
eurorder@probes.nl

Technical Assistance: 9:00 to 16:30 (Central European Time)
Phone: +31-71-5233431 ● Fax: +31-71-5241883
eurotech@probes.nl

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