PhosphoWorks™ Fluorimetric Pyrophosphate Assay Kit

Blue Fluorescence with Enhanced Selectivity

Ordering Information	*Storage Conditions*	*Instrument Platform*
Product Number: 21614 (200 tests)	Keep in freezer and protect from light	Fluorescence microplate readers

Introduction

Pyrophosphate (PPi) are produced by a number of biochemical reactions, such as ATP hydrolysis, DNA and RNA polymerizations, cyclic AMP formation by the enzyme adenylate cyclase and the enzymatic activation of fatty acids to form their coenzyme A esters. AAT Bioquest's PhosphoWroks™ Fluorimetric Pyrophosphate Assay Kits provide the most robust spectrophotometric method for the measurement of pyrophosphate. It uses our proprietary fluorogenic pyrophosphate sensor that has its fluorescence intensity proportionally dependent upon the concentration of pyrophosphate. Our assay is much easier and more robust than enzyme-coupling pyrophosphate methods, which require at least two enzymes for their pyrophosphate detections. Due to its direct measurement of pyrophosphate, this kit is ideal for screening inhibition or activities of enzymes that consume or generate pyrophosphate with enhanced selectivity to pyrophosphate. The assay is an optimized mix-and-read assay and can be performed in a convenient 96-well or 384-well microtiter-plate format. The kit provides all the essential components for assaying pyrophosphate.

Kit Components

Components	Amount
Component A: Assay Buffer	1 bottle (25 mL)
Component B: PPi Sensor	1 vial (lyophilized powder)
Component C: Pyrophasphate Standard	1 mL (50 mM)
Component D: DMSO	1 vial (100 µL)

Assay Protocol (for one 96-well plate)

Brief Summary

Prepare pyrophosphate standards (50 µL) and/or test samples (50 µL) → Add Assay solution (50 µL) → Incubate at room temperature for 10 to 30 minutes → Monitor fluorescence intensity at Ex/Em = 370/470 nm

1. Prepare assay solution:

1.1 Thaw all the four components at room temperature before use.

1.2 Prepare 200X PPi Sensor Stock Solution: Add 50 µL of DMSO (Component D) into the vial of PPi Sensor (Component B) to make 200X PPi Sensor Stock Solution.
Note: 25 µL of the PPi Sensor Stock Solution is enough for one 96-well plate. The unused PPi Sensor Stock Solution should be divided into single-use aliquots. Store at -20°C and protect from light.

1.3 Prepare Assay Solution: Add 25 µL of 200X PPi Sensor Stock Solution (from Step 1.2) to 5 mL of Assay Buffer (Component A), and mix them well.
Note: Due to the high sensitivity of this assay to PPi, it is important to use PPi-free labware and reagents.

2. Prepare serially diluted pyrophosphate standards and test samples:

2.1 Prepare 1 mM Pyrophosphate Standard Solution: Add 10 µL of 50 mM Pyrophosphate Standard (Component C) into 490 µL of Assay Buffer (Component A), or buffer of your choice (preferably 50 mM Hepes buffer, pH 7) to make 1 mM pyrophosphate standard solution.

2.2 Take 200 µL of 1mM pyrophosphate standard solution to perform 1:3 serial dilutions to get 300, 100, 30, 10, 3, 1 and 0 µM serially diluted pyrophosphate standards with Assay Buffer (Component A).

2.3 Add serially diluted pyrophosphate standards and/or pyrophosphate-containing test samples into a solid black 96-well microplate as described in Tables 1 and 2.

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Table 1 Layout of pyrophosphate standards and test samples in a solid black 96-well microplate

BL	BL	TS	TS	….	….						
PS1	PS1	….	….	….	….						
PS2	PS2										
PS3	PS3										
PS4	PS4										
PS5	PS5										
PS6	PS6										
PS7	PS7										

Note: PS = Pyrophosphate Standard, BL = Blank Control, TS = Test Sample.

Table 2 Reagent composition for each well

Pyrophosphate Standards	**Blank Control**	**Test Sample**
Serial Dilutions*: 50 µL	Assay Buffer: 50 µL	50 µL

*Note: *Add serially diluted pyrophosphate standards from 0.3 µM to 100 µM into wells from PS1 to PS7.*

3. Run pyrophosphate assay:

3.1 Add 50 µL/well of Assay Solution (from Step 1.3) to the wells of pyrophosphate standards, blank control, and test samples. *Note: For a 384-well plate, add 25 µL of sample and 25 µL of Assay Solution into each well.*

3.2 Incubate at room temperature for 10 to 30 minutes.

3.3 Monitor the fluorescence increase at Ex/Em = 370/470 nm using a fluorescence plate reader

Data Analysis

The fluorescence in blank wells (with the assay buffer only) is used as a control, and is subtracted from the values for those wells with the pyrophosphate reactions. A pyrophosphate standard curve is shown in Figure 1. *The fluorescence background may increase with time, thus it is important to subtract the fluorescence intensity value of the blank wells for each data point.*

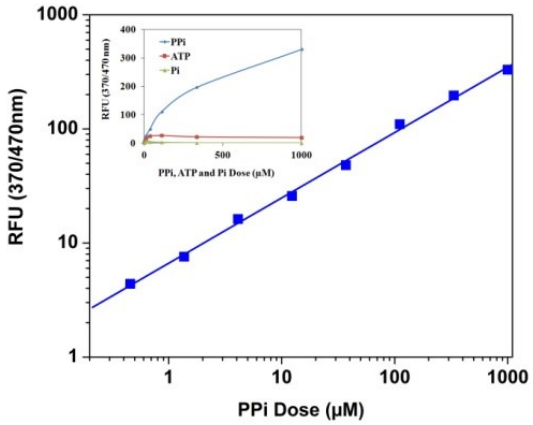

Figure1. Pyrophosphate, ATP and phosphate dose responses were measured with the PhosphoWorks™ Fluorometric Pyrophosphate Assay Kit in a solid black 96-well plate using a fluorescence microplate reader. As low as 1 µM (100 picmoles/well) pyrophosphate can be detected with 10 minutes incubation.

References

1. Zhou M, Diwu Z, Panchuk-Voloshina N and Haugland RP. (1997) A Stable Nonfluorescent Derivative of Resorufin for the Fluorometric Determination of Trace Hydrogen Peroxide: Applications in Detecting the Activity of Phagocyte NADPH Oxidase and Other Oxidases Anal Biochem 253, 162-168.
2. Mohanty, JG, Jaffe JS, Schulman E S and RaibleDG. (1997) A highly sensitive fluorescent micro-assay of H_2O_2 release from activated human leukocytes using a dihydroxyphenoxazine derivative. *J. Immunol. Methods* 202: 133-141.