Technical Bulletin

Pyrophosphate (PPi) Assay Kit (Fluorometric/Colorimetric)

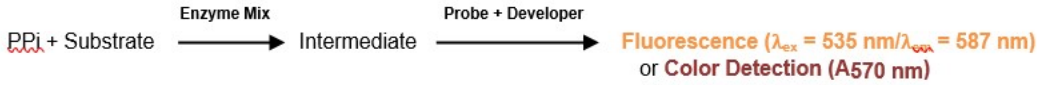
Catalog Number MAK386

Product Description

Inorganic pyrophosphate (diphosphate, pyrophosphoric acid or PPi) is a small molecule formed during a number of biochemical reactions or required as a substrate in other reactions. Enzymatic reactions that generate PPi include ATP hydrolysis, DNA and RNA polymerization, cyclic AMP formation, and enzymatic activation of fatty acids to form their Coenzyme A esters. Conversely, enzymes that utilize PPi may include cyclases, hydrolases, and ligases. Measurement of inorganic pyrophosphate (PPi) in plasma, serum and other biologic fluids is important in studies of bone metabolism, renal stone disease, and certain types of arthritis.

The Pyrophosphate (PPi) Assay Kit provides a fast, convenient and ultrasensitive method for determination of free inorganic pyrophosphate levels in biological material. PPi is detected through a series of reactions which utilize a proprietary enzyme mix and probe, generating a stable product that can be quantified either colorimetrically or fluorometrically. Generated fluorescence (λ_{Ex} = 535 nm/λ_{Em} = 587 nm) or color (570 nm) intensities are directly proportional to the concentrations of pyrophosphate, enabling precise measurements. Monomeric inorganic phosphate (Pi) does not interfere with the assay. The kit can detect as little as 1.8 µM PPi in plasma and serum samples.

The kit is suitable for the determination of PPi concentration, monitoring pyrophosphate release by a variety of enzymes, and for screening inhibitors of enzymes that consume pyrophosphate. Suitable samples include plasma, serum and other biological fluids, cell culture extracts and tissue lysates.



Components

The kit is sufficient for 50 colorimetric or 100 fluorometric assays in 96-well plates.

- PPi Assay Buffer — 25 mL
  Catalog Number MAK386A
- PPi Buffer Supplement — 200 µL
  Catalog Number MAK386B
- PPi Substrate — 2 x 1 vial
  Catalog Number MAK386C
- PPi Enzyme Mix — 200 µL
  Catalog Number MAK386D
- PPi Developer — 1 vial
  Catalog Number MAK386E
- PPi Probe — 200 µL
  Catalog Number MAK386F
- PPi Standard (1 mM) — 200 µL
  Catalog Number MAK386G

Reagents and Equipment Required but Not Provided

- Pipetting devices and accessories (including multichannel pipettor)
- 96 well clear flat-bottom plate. Cell culture or tissue culture treated plates are **not** recommended.
- Fluorescence or spectrophotometric multiwell plate reader, capable of 37 °C temperature setting
- Refrigerated microcentrifuge capable of RCF ≥10,000 × *g*
- Dounce tissue grinder set (Catalog Number D9063 or equivalent)
- Corning® Spin-X® UF concentrators (Catalog Number CLS431478)

Precautions and Disclaimer

For Research Use Only. Not for use in diagnostic procedures. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Storage/Stability

The kit is shipped on wet ice. Store components at -20 °C protected from light.

Preparation Instructions

Briefly centrifuge vials before opening.

PPi Buffer Supplement and PPi Enzyme Mix: Store at -20 °C. Thaw and keep on ice while using.

PPi Substrate: Completely dissolve one vial with 100 µL of purified water and store at -20 °C. Use within one month of reconstitution.

PPi Developer: Completely dissolve with 220 µL of PPi Assay Buffer, aliquot and store at -20 °C. Use within one month of reconstitution.

PPi Assay Buffer, PPi Probe and PPi Standard (1 mM): Warm to room temperature before use. Store at -20 °C. Use within one month after opening.

Procedure

Sample and Positive Control Preparation

1. Centrifuge blood, plasma, and serum samples for 10 minutes at 10,000 × *g* at 4 °C and collect the supernatant.
2. Filter pre-cleared supernatant through a 10 kDa MWCO spin column concentrator (e.g., Corning Spin-X UF concentrator) for 10 minutes at 10,000 × *g* at 4 °C. Use the deproteinized filtrate for analysis.
3. Cells and tissues can be extracted directly in PPi Assay Buffer by mechanical disruption, liquid homogenization, sonication, freeze/thaw cycles, manual grinding, or lysed by your method of choice.
4. Add 2-50 µL of sample into a clear 96-well plate and adjust the total volume to 50 µL with PPi Assay Buffer. For unknown samples, test several doses to ensure the readings are within the Standard Curve range.
5. For samples with significant background, prepare parallel Sample Background Control(s) containing the same amount of sample as in the test wells.
6. Endogenous compounds may interfere with the reaction. To ensure accurate measurement of PPi in the test wells, we recommend preparing parallel Sample(s) and spiking these with a known amount of PPi Standard within the standard curve range.

Colorimetric Standard Curve Preparation

Using undiluted PPi Standard (1 mM), prepare PPi Standards according to Table 1. Mix well.

Table 1.
Preparation of PPi Standards for Colorimetric Assay

Well	1 mM PPi Standard	PPi Assay Buffer	PPi (nmol/ well)
1	0 µL	50 µL	0
2	2 µL	48 µL	2
3	4 µL	46 µL	4
4	6 µL	44 µL	6
5	8 µL	42 µL	8
6	10 µL	40 µL	10

Fluorometric Standard Curve Preparation (0-1 nmol range)

Prepare a 100 µM PPi Standard Solution by diluting the PPi Standard (1 mM) 1:10 in PPi Assay Buffer. Prepare PPi Standards according to Table 2. Mix well.

Table 2.
Preparation of PPi Standards for Fluorometric assay (0-1 nmol range)

Well	0.1 mM PPi Standard	PPi Assay Buffer	PPi (pmol/ well)
1	0 µL	50 µL	0
2	2 µL	48 µL	200
3	4 µL	46 µL	400
4	6 µL	44 µL	600
5	8 µL	42 µL	800
6	10 µL	40 µL	1000

Fluorometric Standard Curve Preparation (0-0.1 nmol range)

Prepare a 100 µM PPi Standard Solution by diluting the PPi Standard (1 mM) 1:10 in PPi Assay Buffer. Further dilute the 100 µM PPi Standard Solution 1:10 in PPi Assay Buffer to generate a 10 µM PPi Standard Solution. Prepare PPi Standards according to Table 3. Mix well.

Table 3.
Preparation of PPi Standards for Fluorometric assay (0-0.1 nmol)

Well	0.1 mM PPi Standard	PPi Assay Buffer	PPi (pmol/ well)
1	0 µL	50 µL	0
2	2 µL	48 µL	20
3	4 µL	46 µL	40
4	6 µL	44 µL	60
5	8 µL	42 µL	80
6	10 µL	40 µL	100

Reaction Mix

1. Mix enough reagents for the number of assays to be performed.
   a. For each well containing Sample (S), Spiked Sample, or Standard, prepare 50 µL of Reaction Mix according to Table 4 (Colorimetric) or Table 5 (Fluorometric). Mix well.
   b. For each Sample Background Control well, prepare Background Control Mix according to Table 4 (Colorimetric) or Table 5 (Fluorometric). Mix well.

Table 4.
Preparation of Colorimetric Reaction Mix

Reagent	Reaction Mix	Background Control Mix
PPi Assay Buffer	30 µL	34 µL
PPi Buffer Supplement	4 µL	4 µL
PPi Substrate	4 µL	4 µL
PPi Enzyme Mix	4 µL	-
PPi Developer	4 µL	4 µL
PPi Probe	4 µL	4 µL

Table 5.
Preparation of Fluorometric Reaction Mix

Reagent	Reaction Mix	Background Control Mix
PPi Assay Buffer	40 µL	42 µL
PPi Buffer Supplement	2 µL	2 µL
PPi Substrate	2 µL	2 µL
PPi Enzyme Mix	2 µL	-
PPi Developer	2 µL	2 µL
PPi Probe	2 µL	2 µL

2. Add 50 µL of the appropriate Reaction Mix or 50 µL Background Control Mix to their respective sample wells.

Measurement

For colorimetric assay, incubate the plate, protected from light, for 30 minutes at 37 °C. Measure absorbance at 570 nm (A_{570}).

For fluorometric assay, incubate the plate for 60 minutes, protected from light, at 37 °C. Measure fluorescence at λ_{Ex} = 535 nm/ λ_{Em} = 587 nm.

Results

1. Subtract the 0 PPi Standard reading (RFU or A_{570}) from all the Standard readings and plot the PPi standard curve.
2. If Sample Background Control readings (RFU or A_{570}) are significant, subtract the Sample Background Control reading from Sample readings.
3. For unspiked Samples, apply the background-corrected absorbance/ fluorescence readings (RFU or A_{570}) to the standard curve to obtain B nmol of PPi in the sample well:

Pyrophosphate (PPi) Concentration (nmol/µL or mM) =

$$(B/V) \times D$$

where:

B = PPi amount from the standard curve (in nmol)

V = Volume of the sample used in the reaction (in µL)

D = Sample Dilution Factor (if applicable).

4. For spiked samples, calculate B by subtracting the Background-corrected Sample reading (RFU or A_{570}) from Background-corrected Spiked Sample reading (RFU or A_{570}):

PPi amount in sample for Spiked Sample (B) =

$$\frac{\text{Sample}_{\text{Corrected}} \times \text{PPi Spike (nmol)}}{[\text{Spiked Sample}_{\text{Corrected}} - \text{Sample}_{\text{Corrected}}]}$$

Note: PPi MW= 446.06 g/mol
(1 nmol PPi = 446.06 ng)

Figure 1.
Typical Fluorometric Pyrophosphate (PPi) standard curve

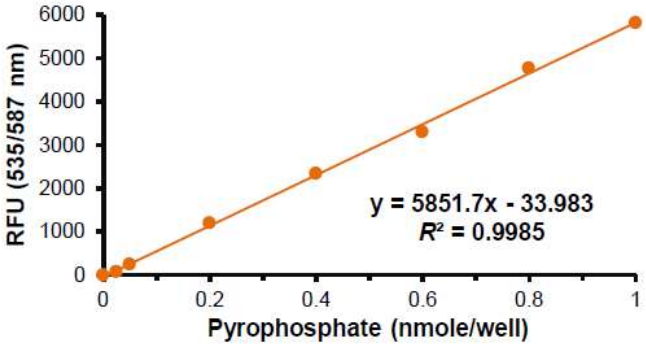


Figure 2.
Typical Colorimetric Pyrophosphate (PPi) standard curve

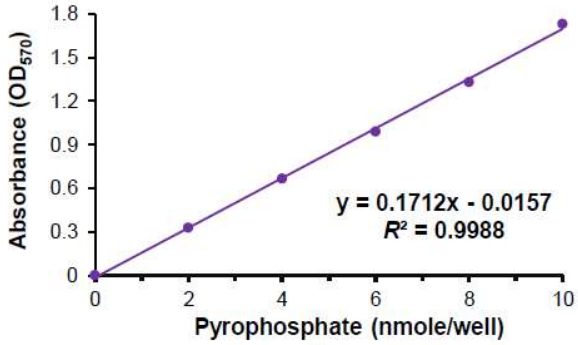


Figure 3.
Quantification of pyrophosphate in 20 µL deproteinized undiluted human serum and pooled plasma. Tested according to kit protocol.

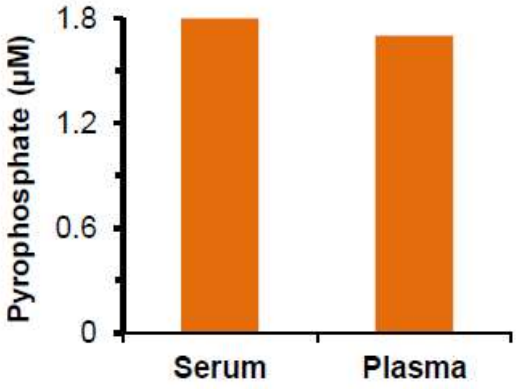


Figure 4.
Spike and recovery in 20 µL of normal human plasma. Plasma samples were spiked with 0.06 nmol of PPi Standard and assayed according to kit protocol, yielding 96% PPi recovery.

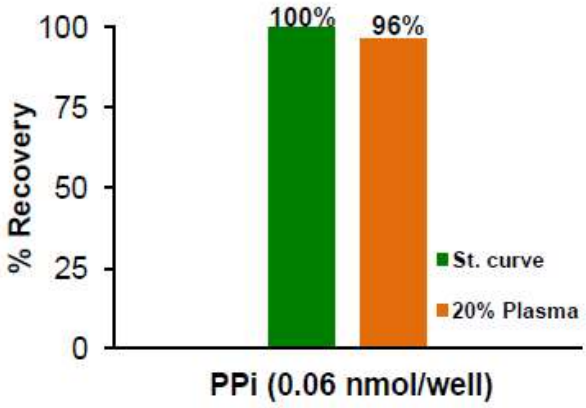
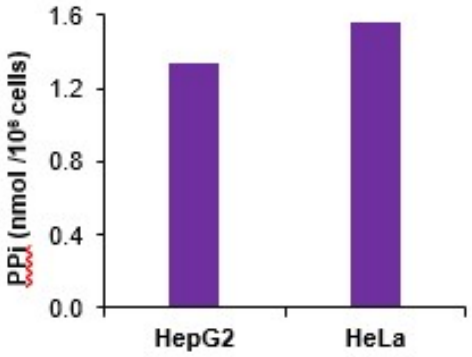


Figure 5.
Pyrophosphate measured in HepG2 and HeLa cell lysates according to kit protocol. Cells were extracted directly in the Assay Buffer.



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