Technical Bulletin

Pyridoxal 5'-phosphate (Vitamin B_6) Fluorometric Assay Kit

Catalot Number MAK425

Product Description

Pyridoxal 5'-phosphate (PLP), the active form of Vitamin B_6 is a cofactor in a variety of metabolic reactions including transamination, decarboxylation, racemization, etc. Vitamin B_6 is comprised of pyridoxine, pyridoxamine and pyridoxal, which during metabolism are converted to the enzymatically active form, namely PLP. Vitamin B_6 is necessary for the synthesis of neurotransmitters, hemoglobin in red blood cells, fat metabolism etc. Vitamin B6 deficiency can lead to nervous disorders, peripheral neuropathy, anemia, seizures etc.

The Pyridoxal 5'-phosphate (Vitamin B_6) Fluorometric Assay Kit provides a quick, specific and easy method for measuring PLP concentrations in a wide variety of samples. In this assay, PLP reacts with a PLP-dependent enzyme which converts the substrate into an intermediate that further reacts with a probe to produce a strong fluorometric signal (λ_{Dx} = 535 nm/ λ_{Em} = 587 nm). The kit is simple, easy to use, sensitive and adaptable to high-throughput applications. The assay can detect as little as 0.2 pmol/well of PLP in biological samples.

The kit is suitable for the measurement of PLP in biological samples (e.g., serum and mammalian tissue) and beverages, and for the analysis of Vitamin B_6 intake on serum.

Components

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

- PLP Assay Buffer — 25 mL
  Catalog Number MAK425A
- PLP Substrate — 1 vial
  Catalog Number MAK425B
- PLP Enzyme Mix — 1 vial
  Catalog Number MAK425C
- PLP Developer 1 — 1 vial
  Catalog Number MAK425D
- PLP Developer 2 — 1 vial
  Catalog Number MAK425E
- PLP Probe — 200 µL
  Catalog Number MAK425F
- PLP Standard — 1 vial
  Catalog Number MAK425G

Reagents and Equipment Required but Not Provided

- Pipetting devices and accessories (e.g., multichannel pipettor)
- Fluorescence multiwell plate reader
- Black flat-bottom 96-well plates. Cell culture or tissue culture treated plates are **not** recommended.
- Refrigerated microcentrifuge capable of RCF ≥13,000 × *g*
- Dounce tissue grinder set (Catalog Number D9063 or equivalent)
- Corning® Spin-X® UF concentrators (Catalog Number CLS431478)
- Bicinchoninic Acid Kit for Protein Determination (Catalog Number BCA1 or equivalent)

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 small vials prior to opening.

PLP Assay Buffer: Warm to room temperature prior to use. Chill an appropriate amount of PLP Assay Buffer for use in Sample Preparation. Store at 2-8 °C or -20 °C.

PLP Substrate, PLP Developer 1 and PLP Developer 2: Reconstitute each vial with 220 µL of PLP Assay Buffer. Aliquot and store at -20 °C. Keep on ice while in use. Avoid freeze/thaw cycles. Use within two months after reconstitution.

PLP Enzyme Mix: Reconstitute with 220 µL of PLP Assay Buffer. Aliquot and store at -80 °C. Keep on ice while in use. Avoid freeze/thaw cycles. Use within two months after reconstitution.

PLP Probe: Ready to use as supplied. Warm to room temperature prior to use. Store at 4°C or -20 °C, protected from light.

PLP Standard: Reconstitute with 200 µL of purified water to make a 10 µM stock PLP Standard solution. Store the stock 10 µM PLP Standard solution at -20 °C, away from light.

Procedure

All samples and standards should be run in duplicate.

Sample Preparation

Tissue Samples

Rapidly homogenize tissue (~10 mg) in 100 µL of ice-cold PLP Assay Buffer with a Dounce Tissue Homogenizer. Keep on ice for 10 minutes. Proceed with "All Sample Types" preparation.

All Sample Types

1. Centrifuge the sample at 13,000 × *g*, 4 °C for 10 minutes to remove the precipitate from the liquid.
2. Collect the supernatant and add 200-500 µL of the supernatant into a 10 kDa Spin Column such as Corning® Spin-X® UF concentrator.
3. Centrifuge the sample at 13,000 × *g*, 4 °C for 20 minutes and collect the filtrate for the assay.
4. For tissue samples, determine protein concentration via BCA protein determination.
5. In a 96-well black plate, add 2-50 µL of the pretreated, filtered sample(s) labeled as Sample. PLP varies over a wide range for different Samples. For unknown samples, perform a pilot experiment with several dilutions to ensure that the

readings are within the Standard Curve range.
Note: Matrix effect can develop in serum samples. Do not use more than 10 µL of human serum per well. For normal human serum, average PLP concentration ranges around 10-200 nM.

6. Adjust the total volume in each Sample well to 50 µL with PLP Assay Buffer.
7. Add 50 µL of PLP Assay Buffer into another well labeled as Blank.

Standard Curve Preparation

Prepare a 100 nM PLP Standard solution by diluting 10 µL of the reconstituted 10 µM PLP Standard stock solution into 990 µL of purified water. Prepare Standards according to Table 1. Mix well.

Table 1.
Preparation of PLP Standards

Well	100 nM PLP Standard	PLP Assay Buffer	PLP (fmol/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

Working Enzyme Mix

Note: PLP Enzyme Mix is a suspension, vortex the tube **every time** before adding to the Working Enzyme Mix.

1. Mix enough reagents for the number of assays to be performed. For each well, prepare 20 µL of Working Enzyme Mix according to Table 2. Mix well.

Table 2.
Preparation of Working Enzyme Mix

Reagent	Volume
PLP Enzyme Mix (vortexed well)	2 µL
PLP Assay Buffer	18 µL

2. Add 20 µL of the Working Enzyme Mix to each well containing Standard, Blank, and Sample(s). Mix well
3. Incubate the plate for 30 minutes at 25 °C, protected from light.

Detection Mix

1. Prepare a 5× dilution of the PLP Probe with PLP Assay Buffer. (i.e., add 5 µL of PLP Probe into 20 µL of PLP Assay Buffer).
2. Mix enough reagents for the number of assays to be performed. For each well, prepare 30 µL of Detection Mix according to Table 3. Mix well.

Table 3.
Preparation of Detection Mix

Reagent	Volume
PLP Assay Buffer	22 µL
PLP Substrate	2 µL
PLP Developer 1	2 µL
PLP Developer 2	2 µL
Diluted PLP Probe	2 µL

3. Add 30 µL of the Detection Mix to each well containing Standard, Blank, and Sample(s). Mix well.

Measurement

Immediately measure the fluorescence (RFU) (λ_{Ex} = 535 nm/λ_{Em} = 587 nm) in a plate reader in kinetic mode every 30 seconds for 30 minutes.

Results

1. Subtract 0 Standard readings from all Standard readings.
2. Plot the RFU vs time.
3. Obtain the initial slope (RFU/min).
4. Plot the initial slope against the PLP amounts in each well and obtain the PLP Standard Curve.
5. Obtain the corrected Sample (RFU_{CS}) readings by subtracting the Blank (RFU_{BL}) reading from Sample (RFU_S) readings:

$$(RFU_{CS} = RFU_S - RFU_{BL})$$

6. Plot RFU_{CS} vs time and use the linear portion of the curve to determine the slope of the kinetic curve.
7. Compare the slope against the PLP Standard Curve to obtain the amount of PLP in the wells (B).

   Concentration of PLP in fluid sample (fmol/μL or nM) =

   (B/V) × D

   Concentration. of PLP in tissue sample (fmol/μg or pmol/mg) =

   (B/(V × P)) × D

where

B = Amount of PLP calculated from the Standard Curve (in fmol)

V = Volume of Sample added to the well (in μL)

D = Sample dilution factor (if applicable; D = 1 for undiluted Samples)

P = Concentration of protein (in μg/μL)

Figure 1.
Typical PLP Standard Curve

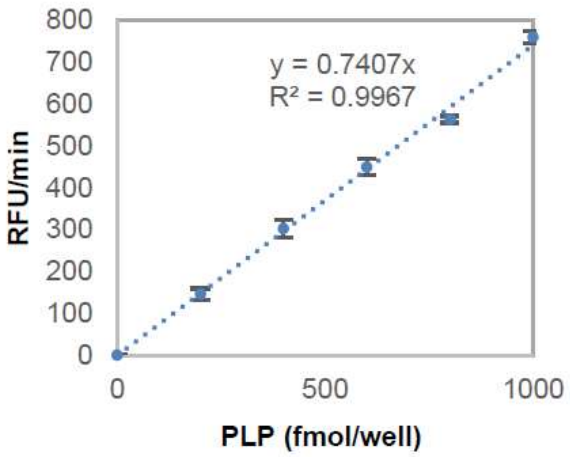


Figure 2.
Corrected kinetic curves for Blank, Orange Juice, Human serum, and Rat liver lysate.

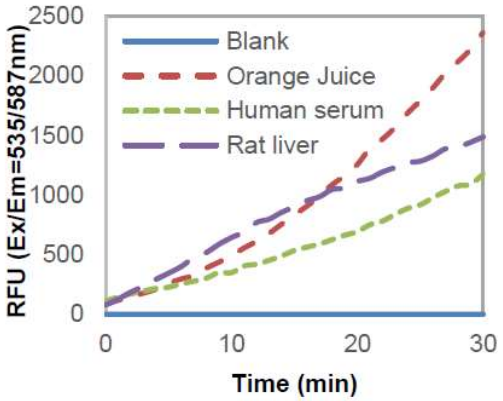
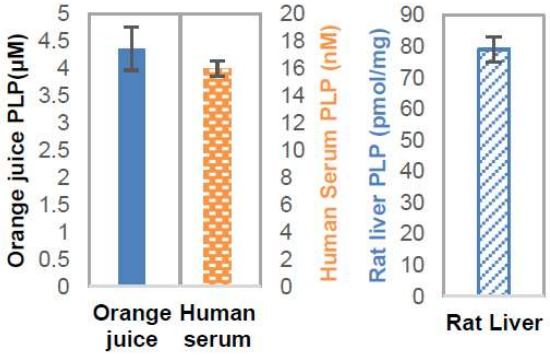


Figure 3.
Estimation of PLP in Orange juice (5 μL of 100× dilution), Human serum (4 μL) and Rat liver lysate (5 μL of 10× dilution).
PLP concentrations were 4.36 ± 0.40 μM in orange juice, 15.98 ± 0.58 nM in human serum and 79.0 ± 4.0 pmol/mg in Rat liver lysate samples. Assays were performed following the kit protocol.



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