CELL TECHNOLOGY

Fluoro Phos

Fluorescent Phosphate Detection Kit

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Notes:

I. Introduction:

Phosphorus is essential for multiple and diverse biological functions, including cellular signal transduction, mineral meatbolism, and energy exchange. Although >80% of total body phosphorus is stored in bone and teeth, intracellular phosphorus exisits in the form of organic compunds such as ATP and as free anions like $H_3PO_4^{3-}$, which are commonly referred to as phosphate. Serum phosphorus primarily occurs in the form of inorganic phosphate, which is maintained within the physiological range by regulation of dietary absorption,bone formation, and renal excretion, as well as equilibrium with intracellular stores. Elevated levels of serum phosphate may be associated with a higher risk of death and adverse cardiovascular outcomes in people with prior myocardial infarction, and is the subject of large amount of research. Rapid and easy quantification of serum phosphate in experimental samples may prove clinically useful.

Applications:

- Quantitation of phosphate anion in blood, plasma and serum samples.

II. Assay Principle:

Cell Technology's Phosphate detection kit provides a simple, **one-step** fluorimetric or colorimetric method for determination of **phosphate** in serum and plasma samples. The assay is based on an enzyme-coupled reaction that detects inorganic phosphate. The substrate is converted to Hydrogen peroxide in presence of inorganic phosphate in a coupled enzymatic reaction. The Hydrogen Peroxide then reacts with the detection reagent in a 1:1 stoichiometry in presence of Horse Radish Peroxidase to produce the stable fluorescent product.

Substrate+ **Pi**

↓ Coupled enzyme reaction

H_2O_2

↓ Horse Radish Peroxidase + Detection Reagent

Fluorescent Product

Lambda $_{max}$ 570nm. Lambda$_{ex/em}$ 535/585nm.

Colorimetric assay can be read on a spectrophotometer at 570nm.
Fluorescence is measured at excitation 530-570nm and emission 585-600nm.

III. Storage:

Store Part 1 of the kit frozen at -20°C upon arrival.
The Detection Reagent (Part # 4025) should be protected from light.
To avoid repeated freeze/thaw cycles, prepare aliquots and freeze.

Allow reagents to warm to room temperature, and spin down vials briefly to ensure contents are not lost in caps.

Store Part 2 (Part # 3059: Reaction buffer, Part #3061: sample diluent, Part #3060: Phosphate standard and Part # 6028: HRP) refrigerated at 2-8°C.

IV. Schematic Representation of the Fluoro Phos Assay:

50µL of diluted serum sample/phosphate standard (Part # 3060)
+
50µL of Reaction cocktail (Enzymes + Substrate + Detection Reagent + Reaction Buffer)

Incubate at 37°C for 60 minutes, DARK
Read on plate reader: Excitation: 535nm, Emission: 585nm

V. Warnings and Precautions:

1. **For Research use only. Not for use in diagnostic procedures**.
2. Practice safe laboratory procedures by wearing gloves, protective clothing and eyewear.
3. The reaction is not stable in the presence of thiols (DTT or 2-mercaptoethanol). Keep these reactants below 10µM.
4. **Once the vial of the Detection Reagent (Part # 4025) is opened, it is important that low lighting conditions be used while aliquoting as well as performing the experiment. Direct and prolonged light exposure may increase the background, resulting in compromised linearity.**

VI. Catalog # FLPHOS100-2 (100 assays) Kit contents and Reagent Preparation:

1. **Part # 4025: Detection Reagent:** 1 vial dried down. Store at -20°C. Resuspend vial contents in 60µL of DMSO. Use 10µL of this solution per mL of reaction cocktail prepared. 1mL of Reaction Cocktail is sufficient for 20 tests. Aliquot the resuspended Detection reagent and freeze as single-use portions.

2. **Part # 6026: Enzyme A:** 30 µL @1000U/ml. Store at -20°C as single use aliquots. Use 4µL of this solution per mL of reaction cocktail prepared. 1mL of Reaction Cocktail is sufficient for 20 tests. **Note: Please use P-10 tips (1-10 µL) to be able to reach liquid in the vial.**

3. **Part # 6027: Enzyme B :** 20 µL @1000U/ml. Store at -20°C as single use aliquots. Use 2 µL of this solution per mL of reaction cocktail prepared. 1mL of Reaction cocktail is sufficient for 20 tests. **Note: Please use P-10 tips (1-10 µL) to be able to reach liquid in the vial.**

4. **Part # 6028: Horse radish Peroxidase enzyme:** 30 µL supplied. Please see vial label for concentration**.** Store refrigerated at 2-8°C. Dilute this enzyme to 100U/ml by adding 4.8 µL of the

enzyme to 95.2 µL of 1X Reaction buffer. For each mL of Reaction cocktail to be prepared, use 4 µL of HRP @ 100U/ml. **Note: Please use P-10 tips (1-10 µL) to be able to reach liquid in the vial.**

5. **Part # 3059: 1X Reaction Buffer**: 14mL supplied. Ready to use. Store at 2-8°C.
6. **Part # 3061: Sample Diluent:** 14ml supplied. Ready to use. Store at 2-8°C.
7. **Part # 3060: Phosphate Standard:** 1mL supplied per kit. Store refrigerated at 4°C. Standard is a 1000mg/L solution of PO_4^{-3} in water (10.5mM). Prepare serial dilutions ranging from 200µM to 1.5625 µM in 1X Reaction buffer. Pipette 50 µL of each dilution in triplicate to wells of the 96-well plate.
8. **Part # 7023: 25X Substrate:** The vial contains 1ml of 25X substrate. Aliquot single use portions and store frozen at -20°C. Dilute by adding 40µL of the supplied vial contents to 960µL of 1X Reaction Buffer. Add 10 µL of this per ml of Reaction cocktail prepared.
9. **1 Black 96-well clear bottom plate for fluorescence plate readers.**

VII. Materials required but not supplied:

1. Fluorescence plate reader.
2. Deionized water.

VIII. Sample Preparation:

Phosphate levels in adult human serum and plasma typically range from 0.8-1.5mmoles/L or mM. (2.5 to 5.5 mg/dL). A 1:400 to 1:800 dilution of serum or plasma samples will give RFU values that will fall within the range of the standard curve. The serum/plasma samples should be diluted in the sample diluent provided, Part # 3061 and run along with the standard curve. Pipette 50 µL of the diluted serum samples into wells of the 96-well plate in triplicate.

IX. Assay Protocol:

Phosphate Standard curve:

To a tube containing 904.8µL of reaction Buffer, add 95.2µL of the 10.5mM Phosphate standard to prepare a 1mM phosphate standard solution.

Label eight test tubes 1-8. To tube #1 containing 800µL of 1X Reaction Buffer, add 200µL of 1mM phosphate to give 200µM (tube #1). Next serially dilute the 200µM phosphate 1:2 by adding 500µL of Reaction Buffer + 500µL of 200µM phosphate (tube #1) to prepare 100µM (tube #2). Continue the serial dilutions through tube #7 to give a concentration of 3.125µM in tube #7. The final concentration of phosphate in the reaction well will be half that in the tube.

Tube # 8 is the zero control.

Tube #	Phosphate Concentration in tubes µM	Final Phosphate Concentration in wells. µM
1	200	100
2	100	50
3	50	25
4	25	12.5
5	12.5	6.25
6	6.25	3.125
7	3.125	1.5625
8	0	0

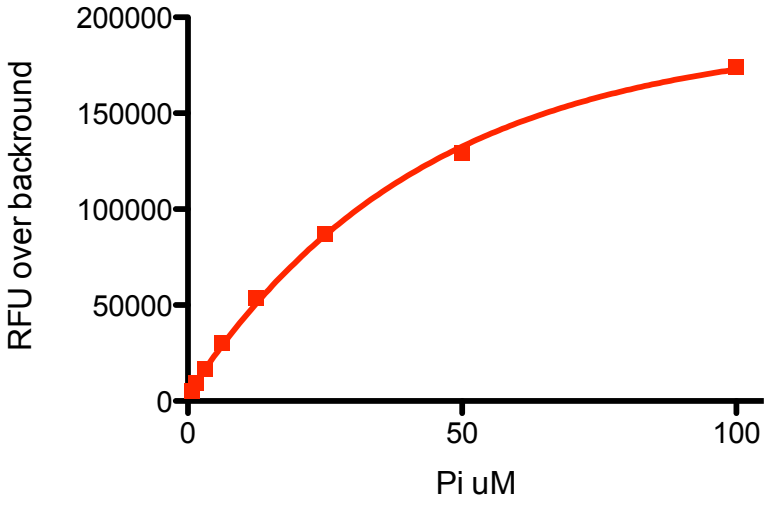

Fig.1 Phosphate standard curve was generated using fluorimetric detection: excitation 535nm and emission 585nm. Incubation time= 60 minutes at 37°C. Standard curve range 1.5625 µM to 100µM. Graph was plotted using 4PL non-linear regression.

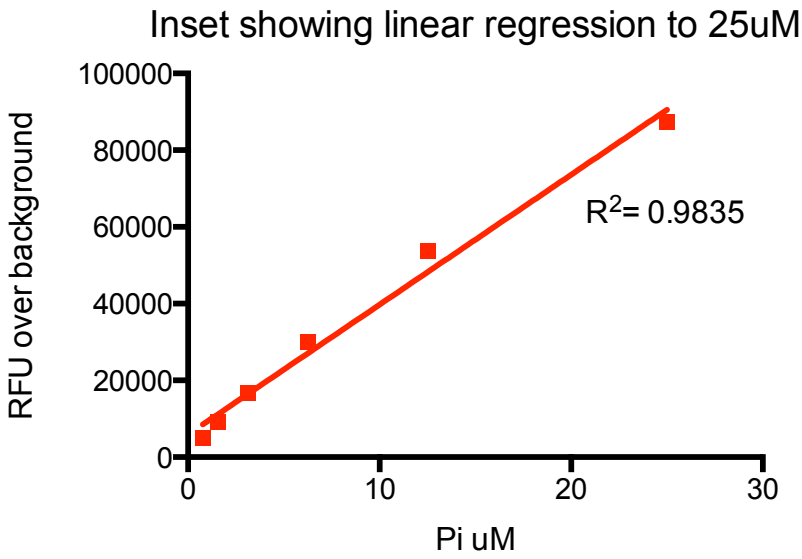

Fig.2 Phosphate standard curve inset:
R^2 =0.9835. Incubation time=60 minutes at 37°C. Standard curve range 1.5625µM to 25µM.
Graph plotted with Linear regression.

Prepare Reaction Cocktail:

For 20 tests, prepare 1ml of Reaction cocktail as follows:
970 µL of 1X Reaction buffer
4 µL of Enzyme A (Part # 6026)
2 µL of Enzyme B (Part # 6027)
4 µL of HRP enzyme @ 100U/ml (Part # 6028 diluted as in Section VI)
10 µL of 1X substrate mix (Part # 7023 diluted as in Section VI)
10 µL of resuspended Detection reagent (Part # 4025 reconstituted as in Section VI)

Assay:

1. Plate 50 µL of phosphate standards and dliuted serum samples on a 96-well microplate (clear-bottom black plate) in triplicate.
2. Add 50 µL of the reaction cocktail prepared above. Mix reaction by shaking on an orbital shaker for 10 seconds.
3. Cover the plate and incubate at 37°C for 1 hour.
4. Equilibrate the plate to room temp for 5 minutes and shake plate for 5-10 seconds.
5. Read fluorescence at excitation 530-570nm and emission 585-600nm using a fluorescence plate reader.

Calculations:

1. Subtract the mean background from the RFU values of the standards/samples. Construct a standard curve by plotting the mean RFU over background on the Y-axis and the phosphate concentration on the X-axis.

2. Determine the slope of the curve by using linear regression analysis. The concentration of phosphate in the samples can be determined from the slope of the phosphate standard curve.

3. Multiply the µM concentrations obtained for the samples by the dilution factors used to get concentration in mmoles/L or mM. Multiply this number by 2 to get the final concentration of phosphate which is twice the concentration in the well.

Spike and Recovery results:

10 µM (final concentration) of the phosphate standard was spiked into the 1:400 and 1:800 human serum dilutions and recoveries were determined using the Fluoro Phos kit.

Serum dilution	Pi concentration µM	Pi concentration µM with 10 µM Pi spike	% Recovery of the spiked phosphate
1:800	8.215	17.562	93.47
1:400	15.803	25.557	97.54

The results also show the 1:2 titration of the serum phosphate levels corresponding to the 1:2 dilution of serum.

The above Pi concentrations were determined by interpolating the unknowns using linear regression analysis of the Pi standard curve.

X. Notes:

1. To adapt this as a colorimetric assay, the standard curve has to be constructed in a range of 0 - 160 µM and calculations done based on the standard curve generated. Absorbance readout at 570nm. Absorbance and fluorescent readouts should be performed on separate plates to avoid quenching effects.

2. The final phosphate concentration will be **twice** the concentration in the well. It is important to remember to multiply the concentrations by 2 as well as the dilution factor used.

XI. References:

1. Mohanty, J.G. *et al* : A highly sensitive fluorescent micro-assay of H_2O_2 release from activated human leukocytes using a dihydroxyphenoxazine derivative, **J.Immunological Methods**, 202, issue 2, P.133-141 (1997)

2. Tonelli, M. et al : Relation between serum phosphate level and cardiovascular event rate in people with coronary disease, **Circulation**,112, P.2627-2633 (2005)