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Pyrophosphate Colorimetric Assay Kit

Product Manual

A0012-01 **50 tests**

A0012-02 **100 tests**

A0012-03 **200 tests**

For research use only
Not intended for human or animal diagnostic or therapeutic uses

PRECAUTIONS:

(1) Read the product manual carefully before use!

(2) All kit components have been formulated and quality controlled. The accuracy, precision, resolution or sensitivity may be severely compromised by any modifications to the kit components or procedures.

(3) Do not use kit or components if it has exceeded the expiration date printed on the kit labels.

(4) Do not mix or substitute reagents or materials from other kit lots or other vendors.

(5) Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

(6) Some components in this kit contain preservative and other chemicals which may cause skin, eye, and respiratory irritation or allergic reaction, avoid breathing mist or fumes. Please wear protective gloves, clothing, Goggles and face shield for safety. Rinse hands thoroughly after handling.

(7) Eyes, mouth or skin, etc. that have been in contact with the reagents should be rinsed immediately with water continuously for at least 15 minutes and get an immediate medical advice.

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INTRODUCTION

Pyrophosphate (Diphosphate, PPi) is a small intermediate molecule formed in a number of biochemical reactions, such as the degradation of NTP, the synthesis of DNA or RNA, etc. PPi can be found in synovial fluid, blood plasma, and urine. PPi may block hydroxyapatite formation. Three molecules (ENPP1, ANK, and TNAP) have been reported to act as the central regulators of pyrophosphate levels. ENPP1 is a type II transmembrane ecto-enzyme that hydrolyzes extracellular ATP into AMP and PPi. ANK is an ion channel mediating intracellular-to-extracellular channeling of pyrophosphate. Recently, ENPP1 has been recently shown to be shed as a soluble ENPP1 which could function intracellularly and might provide the source of pyrophosphate for transport by ANK. TNAP, the PPi hydrolase, further exerts its effects on PPi level by hydrolyzing PPi to phosphate. BLMSAF's Pyrophosphate Colorimetric Assay Kit provides a convenient method to detect Pyrophosphate in a variety of biological samples including animal blood, tissue fluid and cell lysates. In the assay, Pyrophosphate is converted to pyruvate in the presence of Phosphoenolpyruvate. This is followed by a set of enzymatic reactions to generate a colored product with a strong absorbance at 570 nm, which is proportional to the amount of Pyrophosphate present in samples.

The kit is rapid, sensitive and high-throughput adaptable. It can measure Pyrophosphate level lower to 0.5 nmol (5 μM conc., if the sample volume is 100 μL) in various types of biological samples.

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Kit contents & storage conditions

Components	50 tests	100 tests	200 tests	Storage
PPi Lysis Buffer	1 × 30 mL	1 × 60 mL	1 × 120 mL	4 °C
PPi Assay Buffer	1 × 30 mL	1 × 60 mL	1 × 120 mL	4 °C
PPi Developer	2 vials	4 vials	8 vials	-20 °C
PPi Enzyme mix	1 × 3 mL	1 × 6 mL	1 × 12 mL	-20 °C
PPi Probe	1 × 0.3 mL	1 × 0.6 mL	1 × 1.2 mL	-20 °C
PPi Substrate	1 × 1.5 mL	1 × 3 mL	1 × 6 mL	-20 °C
PPi Standard	1 × 0.5 mL	1 × 1 mL	1 × 2 mL	-20 °C

Operation, Storage and stability

All Pyrophosphate Colorimetric Assay Kit components are guaranteed for at least 12 months from the date of purchase before reconstitution when properly stored as followed:

(1) Store kit at -20 °C in the dark immediately upon receipt.

(2) Keep all the components on ice (except PPi probe) and avoid direct exposure of strong light sources during the assay.

(3) PPi Lysis Buffer and PPi Assay Buffer could be stored in 4 °C.

(4) All the other components must be stored at -20 °C.

(5) Reconstituted PPi developer should be used within ONE WEEK, decomposition of the reagent will cause high background signals.

(6) Please ensure that all the bottles and tubes are tightly capped before putting them into refrigerator.

MATERIALS NOT SUPPLIED

The materials are not included in the kit, but will be required for this assay:

(1) PBS

(2) Qualified flat bottom clear 96 well plate with cover or plate sealer

(3) Refrigerated microcentrifuge

(4) Pipettes and pipette tips

(5) Colorimetric microplate reader

(6) Heat block

(7) Vortex

(8) Dounce homogenizer or pestle (if using tissue)

(9) 37 °C incubator

(10) Flake ice maker

(11) Disposable nitrile or neoprene gloves

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TECHNICAL HINTS

(1) Review the protocol completely to confirm that this kit meets your requirements. Please contact our Technical Support with any questions.

(2) Keep enzymes, heat labile components and samples on ice during the assay.

(3) Make sure all buffers and solutions are thawed before starting the experiment.

(4) Samples generating values higher than the highest standard should be further diluted with PPi Assay Buffer.

(5) Avoid foaming or bubbles when mixing or reconstituting components and make sure there should be NOT any bubble in any well of the plate before reading it.

(6) The kit is highly sensitive, please avoid any cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.

(7) Ensure that the plates are properly sealed or covered during incubation step. Make sure you have the qualified 96 well plates for your detection. Variation of the background well to well absorbance severely deteriorates assay accuracy.

(8) To be accurate, the standard samples should be run in the same matrix as the unknown samples.

ASSAY SUMMARY

Sample preparation

Standard curve preparation

Reaction mix preparation

Add reaction mix and incubate at 37 °C for 0.5~2 hours

Measure absorbance at 570 nm

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ASSAY PREPARATION

Briefly centrifuge small vials at low speed prior to opening.

(1) PPi Lysis Buffer: Ready to use as supplied. Store at 4 °C.

(2) PPi Assay Buffer: Ready to use as supplied. Store at 4 °C.

(3) PPi Developer: Reconstitute 1 vial of the PPi Developer in 55 μL of PPi Assay Buffer. Pipette up and down to completely dissolve. Reconstituted PPi developer should be used within A WEEK if stored at -20 °C.

(4) PPi Enzyme mix: Ready to use as supplied. Enzymes are heat labile, always keep on ice while in use. Store at -20 °C.

(5) PPi Probe (DMSO solution): Warm with hand or 37 °C bath for ~1 min to thaw the DMSO solution and put it under ambient conditions (20-30 °C) while in use.

(6) NOTE: DMSO solution tends to be solid below 20 °C, it needs to melt for few minutes at 37 °C. Store at -20 °C and protect from light.

(7) PPi Substrate: Ready to use as supplied. Briefly thaw in hand or 37 °C bath and keep on ice while in use. Store at -20 °C.

(8) PPi Standard: Ready to use as supplied. Briefly thaw in hand or 37 °C bath and keep on ice while in use. Store at -20 °C.

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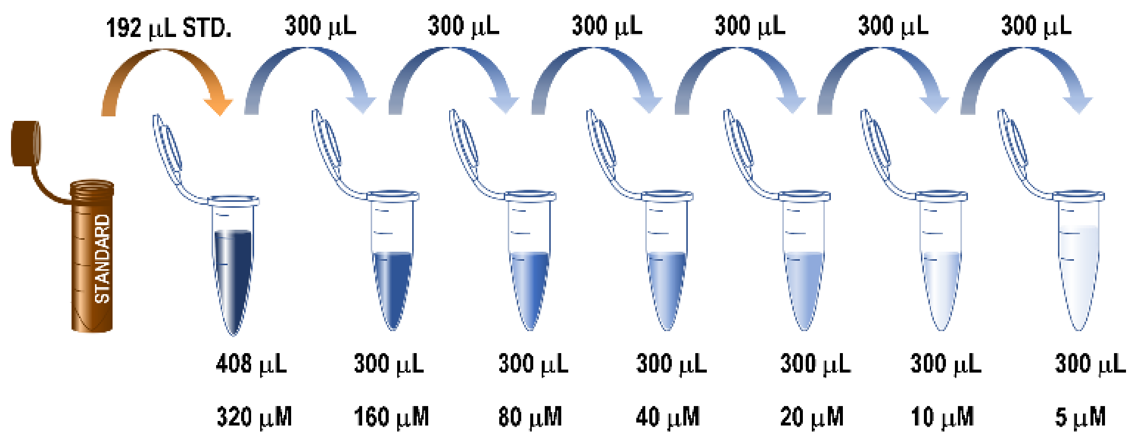
STANDARD PREPARATION

Always prepare a fresh set of standards for each assay. Diluted standard solution is unstable and must be used within 8 hours.

Standard #	Volume of PPi Standard (μL)	PPi Lysis buffer (μL)	Final volume in well (μL)	Final conc. in well (μM)
1	0	300	300	0
2	1.5	298.5	300	5
3	3	297	300	10
4	6	294	300	20
5	12	288	300	40
6	24	276	300	80
7	48	252	300	160
8	96	204	300	320

HINT: practical serial dilution strategy

The above table lists the final concentration and volume required for each standard. **Serial dilution** can be practically used for standard preparation (see figure of next page). The concentration of Pyrophosphate Standard stock solution is 1 mM. Prepare seven 1.5 mL Eppendorf tubes. Pipette 408 μL of PPi Assay Buffer into the 320 μM tube. Pipette 300 μL into the remaining tubes. Use the PPi Standard stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 320 μM serves as the high standard. PPi Assay Buffer serves as the zero standard (0 μM). Each dilution has enough amount of standard to set up duplicate readings (2 × 100 μL).



NOTE: If your sample readings fall out the range of your standard curve, the appropriate dilution adjustments of the samples are necessary.

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SAMPLE COLLECTION & STORAGE

General Sample information:

(1) Several dilutions of your sample might be necessary to ensure the readings are within the standard value range.

(2) Assay samples should be freshly prepared.

(3) If you cannot perform the assay at the same time, please snap freeze the extracted samples in liquid nitrogen and store the samples immediately at -80 °C. Thaw them on ice when you are ready to assay. Be aware however that this might still affect the stability of your samples.

Adherent cell samples:

(1) Culture adherent cells to approximately 80% confluence on 100 mm or 6-well polystyrene tissue culture plates. Cells should be maintained in healthy state.

(2) Aspirate or decant media and keep plates on ice.

(3) Wash cell monolayer gently 3 times with 10 mL ice cold PBS. Aspirate excess PBS.

(4) Add 200~500 μL of PPi Lysis Buffer to each well and swirl to distribute buffer.

NOTE: Please be aware that smaller volume of lysis buffer used will result in higher concentration of Pyrophosphate in the final lysate. The amount of lysis buffer should be empirically determined for each sample type to ensure the efficient lysis as well as an optimal concentration of Pyrophosphate in the lysate.

(5) Scrape the cells with a cell scraper and transfer the lysate to a 1.5 mL micro-centrifuge tube. Carefully pipette the lysate up and down 10~15 times with P200 and vortex vigorously for 10 sec to completely lyse cells. Incubate the lysate on ice for 15 minutes. (Efficient lysis could

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be confirmed by viewing the cells under a microscope).

(6) Centrifuge at 13,000 × g for 10 minutes at 4 °C.

(7) Collect the supernatant (avoiding the pellet) into new microtubes.

(8) Determine the protein concentration with appropriate kit (e. g. the Bicinchoninic acid or Bradford method, etc.).

Suspension cell samples:

(1) Culture cells to a density of 1~2 × 10^6 cells /mL. Cells should be morphologically healthy.

(2) Harvest cells in a conical tube by spinning at 100~300 × g for 5 minutes at room temperature.

(3) Aspirate media and keep cells on ice for all steps.

(4) Wash pellet 3 times with 5 to 10 mL ice cold PBS, spin 300 × g for 5 minutes and aspirate the PBS.

(5) Add 20~100 μL of PPi Lysis Buffer per 1 × 10^6 cells.

NOTE: Pleas be aware that smaller volume of lysis buffer will result in higher concentration of Pyrophosphate in the final lysate. The amount of lysis buffer should be empirically determined for each cell type to ensure efficient lysis as well as an optimal concentration of Pyrophosphate in the lysate.

(1) Carefully pipette the lysate up and down 10~15 times with P200 and vortex vigorously for 10 sec to completely lyse cells. Incubate the lysate on ice for 15 minutes. (Efficient lysis could be confirmed by viewing the cells under a microscope).

(2) Centrifuge at 13,000 x g for 10 minutes at 4 °C.

(3) Collect the supernatant (avoiding the pellet) into new microtubes.

(4) Determine the protein concentration with appropriate kit (e. g. the Bicinchoninic acid or

Bradford method, etc.).

Tissue Samples:

(1) Dissect and harvest the amount of tissue necessary for each assay (initial recommendation = 20~200 mg) into ice-chilled 1.5 mL microcentrifuge tube that contains 1 mL of chilled PBS. The tissue sample should thereafter be on ice preferably.

(2) Close the lid and vortex at maximal speed for 10 sec to wash the dissected tissue to remove blood.

(3) Repeat washing two more times and lyse in 300~500 μL of ice-cold PPi Lysis Buffer in 1.5 mL tube with pestle or homogenize in Dounce homogenizer (if the tissue samples seem to be resistant to lysis under normal conditions).

(4) Centrifuge at 13,000 rpm for 10 minutes at 4 °C and remove an aliquot of the supernatant for protein

(5) Collect the supernatant (avoiding the pellet) into new microtubes.

(6) Determine the protein concentration with appropriate kit (e. g. the Bicinchoninic acid or Bradford method, etc.).

Serum Samples:

Serum can be obtained through clotting whole blood in a microtube or by a specialized serum separator collection tube (SST) before centrifugation for 15 minutes at 1000 × g. Remove serum and assay immediately or store samples at -20 °C and assay within 1 week. Avoid repeated freeze-thaw cycles.

NOTE: Please carefully perform blood collection to avoid the risk of hemolysis or contamination, otherwise this may give false results.

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Plasma Samples:

Collect plasma sample using EDTA or heparin (preferred) as an anticoagulant. Centrifuge for 15 minutes at 1000 × g within 30 minutes of collection. Assay immediately or store samples at -20 °C and assay within 1 week. Avoid repeated freeze-thaw cycles.

NOTE: Please carefully perform blood collection to avoid the risk of hemolysis or contamination, otherwise this may give false results.

Tissue fluid:

Tissue fluid can be assayed immediately without any pretreatment. If some of the fluid samples are not clear, we recommend centrifuging at 13,000 rpm for 10 minutes at 4 °C and collecting the supernatant for subsequent assay or storing at -20 °C within 1 week. Avoid repeated freeze-thaw cycles.

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ASSAY PROCEDURE & DETECTION

(1) Prepare all reagents and working standards as directed in the previous sections.

(2) Set up Reaction wells:

Standard wells = 100 μL standard dilutions.

Sample wells = 100 μL samples.

Background sample wells: 100 μL samples.

NOTE: For statistical reasons, we recommended that all standards, Backgrounds, and samples be assayed in duplicate or triplicate.

(3) Colorimetric Reaction Mixes:

Prepare enough reagents for the number of assays including Standards, Background and Samples to be performed. For each well, prepare 200 μL Colorimetric Reaction Mix containing:

Components	Standard	Background	Sample
PPi Assay Buffer	57 μL	59 μL	57 μL
PPi Enzyme mix	20 μL	20 μL	20 μL
PPi Probe	1 μL	1 μL	1 μL
PPi Substrate	20 μL	20 μL	20 μL
PPi Developer	2 μL	-	2 μL
PPi Standard	100 μL	-	-
Assay sample	-	100 μL	100 μL
TOTAL	200 μL	200 μL	200 μL

HINT:

a) Prepare enough volume of **Master Mix** that omitting PPi Developer [V=98 μL × (N_{sample} × 2 + $N_{standard}$ + 3)], mix by vortex.

b) From the **Master Mix** prepared above, pipette the volume for Standards and Sample [V=98 μL × (N_{sample} + $N_{standard}$ + 1)], and add PPi Developer [V=2 μL × (N_{sample} + $N_{standard}$ + 1)] to the aliquot, mix by vortex.

c) Pipette other aliquot volume of **Master Mix** for background [V=98 μL × (N_{sample} + 1)] and add PPi Assay Buffer [V=2 μL × (N_{sample} + 1)] to the aliquot, mix by vortex.

Add 100 μL of the Colorimetric Reaction Mix prepared above to each corresponding well(s) containing 100 μL of the Standards, Background or Samples. Mix gently. Background reaction mix should be prepared to correct for the background absorbance by omitting the PPi Developer.

(4) Cover with the plate lid or adhesive strip and incubate for 0.5~4 hours on a horizontal orbital microplate shaker in 37 °C incubator. Protect from light. A recommended plate layout with all conditions in duplicate is provided below. For user recording blanks, standards and samples, two blank templates for custom layout have also been printed in next pages.

(5) The color in the wells should change into pink after incubation. The specific incubation time must be empirically determined to ensure optimal assay performance. However, please be aware that too long incubation may cause signal saturation or even attenuation in high concentration wells (including standard wells)

(6) Determine the optical density (O.D.) of each well using a microplate reader set to 570 nm (or the wavelength close to 570 nm).

CALCULATION OF RESULTS

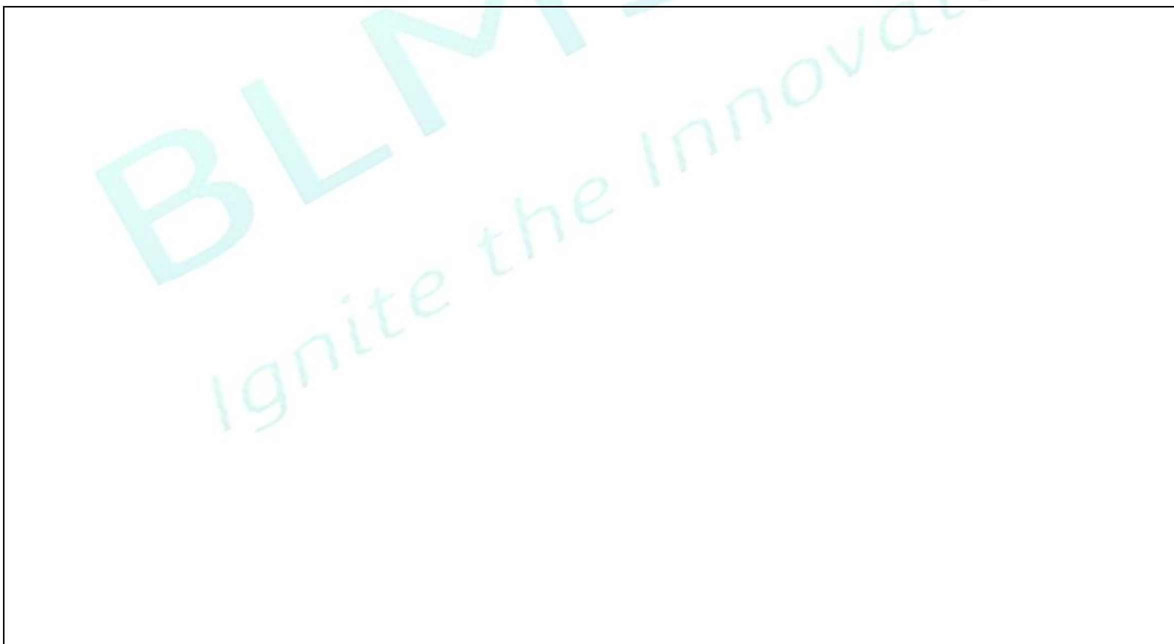
(1) Average the duplicate readings for each Standard and Sample and subtract the corresponding average Sample Background O.D.

(2) Plot the corrected O.D. values for each standard as a function of the final concentration of Pyrophosphate.

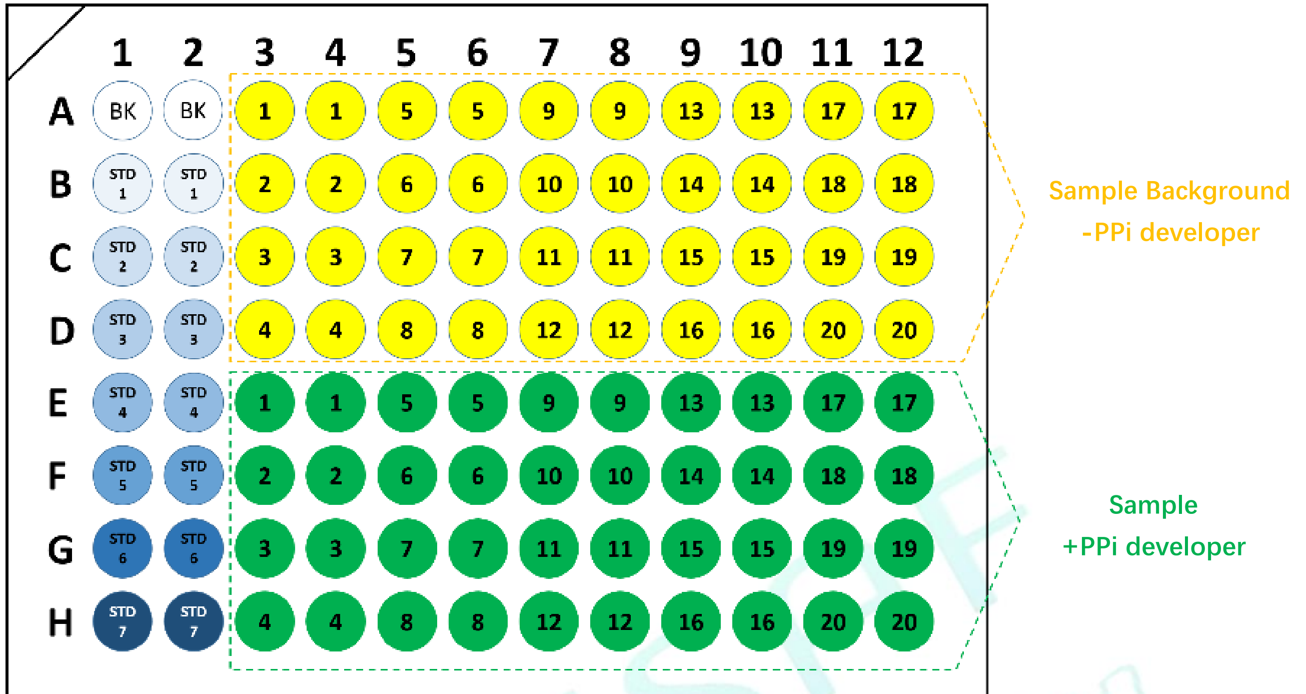
(3) Draw the best smooth curve through these points to construct the standard curve and calculate the trendline equation with plate reader software or Excel (use the equation that provides the highest R^2 value, predicting the perfect fit of the data). Using the equation to calculate the Sample Pyrophosphate concentration. Diluted samples must be multiplied by the dilution factor.

Lot specific standard curve DATA

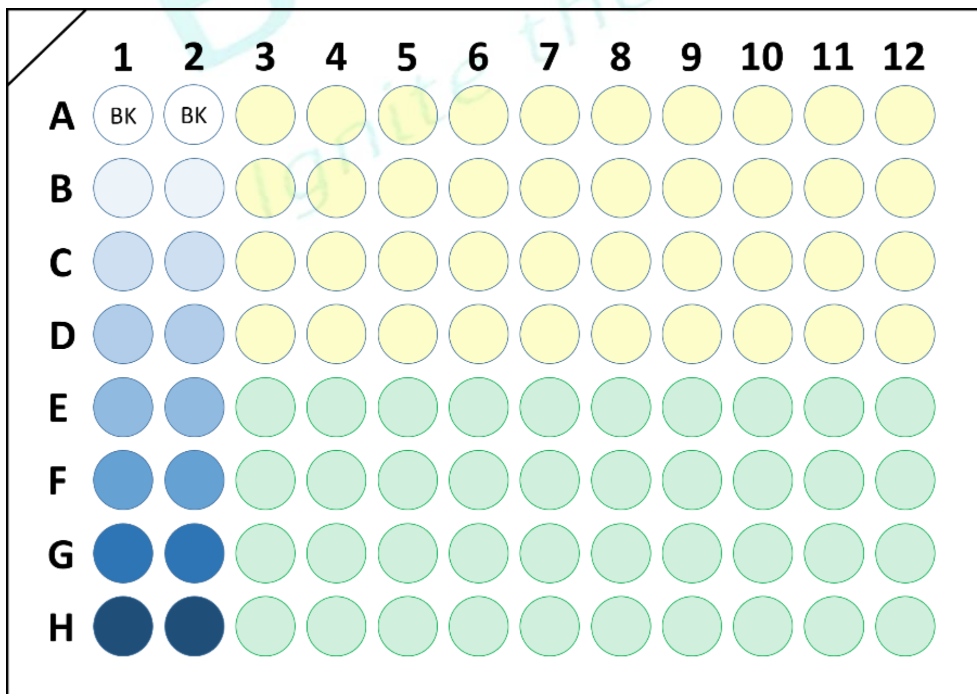
This standard curve is provided for demonstration purpose only (using the kit with same lot number). However, a new standard curve should be generated for each individual assay.



RECOMMENDED PLATE LAYOUT



USER PLATE LAYOUT



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TROUBLESHOOTING GUIDE

This guide helps you to troubleshoot any problems or issues that might occur when using this kit. For further assistance, please contact our technical support at 0755-82192648 or 15323857656.

Assay not working	
Incorrect incubation conditions	Make sure the incubation condition is 37 °C for more than 30 mins.
Plate reader malfunctioned or at incorrect settings	Check the instrument settings and check the wavelength and filter settings.
Misusing of 96-well plate	Please use clear plates for absorbance reading

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Sample with erratic readings	
Plate reader setting at incorrect wavelength	Check the wavelength and filter settings of instrument
Samples and/or assay reagents not homogenized or mixed completely	Carefully homogenize and vortex Increase the number of pipetting.
Formation of foams and/or bubbles in well(s).	Carefully pipetting reagents into 96 well plate. Pipette gently against the wall of the tubes and wells. Do not blow out to eject all liquid at the tip. Remove the foam or bubble by the Defoamer.
Use of inappropriately prepared samples	Fresh samples prepared as indicated in this manual are recommended. Please avoid using samples that are old or prepared with other lysing methods.

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Standard readings do not follow a linear pattern	
Pipetting errors or sequential dilution error in standards or Reaction Mix.	Avoid pipetting small volumes and delayed operation. Change tips between each sample, use 8 or 12-channel pipette and prepare master mix whenever possible.
Air bubbles formed in well.	Carefully pipetting reagents into 96 well plate. Pipette gently against the wall of the tubes and wells. Do not blow out to eject all liquid at the tip. Remove the foam or bubble by the Defoamer.
Standard stocks are at incorrect concentrations	Always prepare fresh standards for each assay.
overextended incubation time	As mentioned above, long incubation may cause the signal attenuation in high concentration wells (including standard wells).

Unanticipated results	
Measured at incorrect wavelength.	Check equipment and filter setting
Samples contain interfering substances.	Please check the sample wells are transparent without any formation of turbidity with naked eyes or microscope. High concentrations of divalent ions, such as Mg^{2+}, Ca^{2+}, etc., will cause the

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	formation of precipitates.
Sample readings are above or below the standard linear range	As mentioned above, please concentrate or dilute samples so as to within the linear range.
Overextended incubation time	Although the incubation time can be empirically determined, too long incubation may cause the color to change from pink to blue-purple, resulting in the signal attenuation in high concentration wells (including standard wells).

Lower/ Higher readings in samples and Standards	
Incorrect incubation time or temperature	Incubation temperature should be 37 °C. However, incubation time should be empirically determined, but never longer than 2 hours. Extended incubation time will cause higher or even saturated readings.
Improperly diluted samples	Samples should be properly diluted to make the readings fall into the range of standard curve. Please increase the cell number or decrease lysis volume If the readings are below the lowest standard.

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Degradation of PPi Developer	PPi Developer is highly unstable if dissolved. Its degradation will cause high background signals. Please reconstituted PPi developer must be used within ONE WEEK even stored at -20 °C or -80 °C.
Inadequate lysis of samples or the cell numbers (tissue size) are highly variable among each sample.	In addition to the observation under microscope, sufficient lysis of sample could also be reflected by protein assay. Protein concentration should be comparable among each sample and each group. Treatments that cause drastic cell loss or tissue necrosis should be avoid.
The potential interfering substance in the sample	Strong oxidants such as hydrogen peroxide, the toxic compound sodium azide, large excess of heavy metal ions (Hg^{+}, Ag^{+}, Pb^{+}, etc.), and divalent metal chelating agent (EDTA, citrates, etc) or any thiol-reducing agent, such as DTT must be excluded from reaction.
The prepared sample is colored	Colored sample with the absorbance near the wavelength of 570 nm will significantly interfered with the readout. Please try to avoid any of those substances.

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After-Sales & Customer Service are ensured by BLMSAF Biotech:

(1) Our products are Quality Guaranteed and Expert Technical Support.

(2) Our products are valid for at least 12 months from date of delivery.

(3) Replacement or refund for products not performing as stated on the product manual.

(4) In-time and professional response to your inquiry within 2 hours.

(5) All suggestions and quality concerns are highly valued to ensure our products meet or exceed the highest standards of quality.

Please customers be noted for limited uses and potentially legal and regulatory Requirements:

(1) All products are "FOR LABORATORY RESEARCH USE ONLY. NOT FOR HOUSEHOLD OR USE IN DIAGNOSTIC PROCEDURES".

(2) Purchaser must determine the suitability, the regulatory requirements of the products for their particular use. Additional terms and conditions may apply.

(3) Products are provided for customer as the end-user and solely for research use, not for any redistribution to any third party.

(4) Please refer to product information on the BLMSAF Biotech website at blmsaf.com and/or on the product manual of the packing slip.

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