Phosfinity

Total Polyphosphate Quantification Kit

AMINOVERSE
EXPLORING ENZYME INNOVATION

Contents

Thank you for your trust and confidence in us and our **Phosfinity – Total Polyphosphate Quantification Kit**.

We always strive to further improve our products, so if you want to give feedback feel free to contact us at: info@aminoverse.com.

1. Introduction

Inorganic polyphosphate (polyP, Figure 1) can be found as a linear polymer of phosphate (P_i) in most living organisms[1]. Its main features revolve around, e.g., energy storage, blood coagulation, channel formation between cell membranes and biofilm formation[2,3]. For studies of the phosphate physiology in organisms, it is important to have a reliable and precise quantification method.

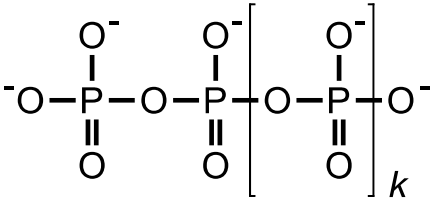

Figure 1: Linear polyphosphate

The new **Aminoverse Total Polyphosphate Quantification Kit** provides a reliable and convenient option to quantify polyP of all chain lengths, even in highly contaminated samples, down to $polyP_2$. The assay's high specificity is enabled by enzymatic polyP hydrolysis, followed by colorimetric P_i detection in an easy two step protocol that can be done in less than 1.5 hours with minimal hands-on time.

The kit is sufficient for 100 reactions.

100 samples can be measured if the kit is used in one pass. The kit requires one negative control measurement and one positive control measurement (i. e. 2 reactions) per day.

2. Key features

- High specificity for polyP due to enzymatic polyP detection
- Quantification in the range of 5 to 200 µM (as monomer) polyP
- Detection of all polyP chain lengths down to $polyP_2$
- Quick colorimetric (2 min) P_i detection. This circumvents artificially high blank measurements due to non-enzymatic hydrolysis of acid-instable phosphorus-containing substances
- Little amount of sample required (e.g., 200 µL of 100 µM polyP)
- No interference from commonly encountered substances

3. Kit components

Phosphate standard (20, 65, 110, 155, 200 µM)	5x 1 mL
Negative control	1 mL
Positive control	1 mL
Enzyme	120 µL
Enzyme reaction buffer	12 mL
Phosphate detection solution A	12 mL
Phosphate detection solution B	1.5 mL

4. Equipment required but not provided

- Clear flat bottom 96 well plate
- (Multi-channel) Micro pipette
- Spectrophotometric multiwell plate reader capable of reading absorbance at 882 nm

5. Storage and stability

The **Enzyme** needs to be stored at **-20 °C** (avoid many freeze/thaw cycles).

Store the remaining kit components at 4-8 °C.

6. Preparation of reagents

Phosphate detection reagent (always prepared freshly)

(50 µL per well, includes negative control, positive control, P_i standards, blanks and samples):

Combine 9 volume parts of solution A and 1 part of solution B (e.g., 45 µL A + 5 µL B). See Table 1 for quick calculation.

Enzyme master mix (always prepared freshly)

(50 µL per sample. NOT for: P_i standards and blanks):

Combine 49 volume parts of enzyme reaction buffer and 1 part of the enzyme solution (e.g. 49 µL buffer + 1 µL enzyme). See Table 2 for quick calculation.

A 20 % excess is recommended to accommodate for loss by using multichannel pipette reservoirs and pipetting error.

Table 1: Preparing the **phosphate detection reagent**.

Wells	Component		Wells	Component	
	A	B		A	B
1	54 µL	6 µL	20	1080 µL	120 µL
2	108 µL	12 µL	30	1620 µL	180 µL
3	162 µL	18 µL	40	2160 µL	240 µL
4	216 µL	24 µL	50	2700 µL	300 µL
5	270 µL	30 µL	60	3240 µL	360 µL
6	324 µL	36 µL	70	3780 µL	420 µL
7	378 µL	42 µL	80	4320 µL	480 µL
8	432 µL	48 µL	90	4860 µL	540 µL
9	486 µL	54 µL	100	5400 µL	600 µL
10	540 µL	60 µL			

Table 2: Preparing the **enzyme master mix**.

Reactions	Buffer	Enzyme	Reactions	Buffer	Enzyme
1	58.8 µL	1.2 µL	20	1176 µL	24 µL
2	117.6 µL	2.4 µL	30	1764 µL	36µL
3	176.4 µL	3.6 µL	40	2352 µL	48 µL
4	235.2 µL	4.8 µL	50	2940 µL	60 µL
5	294.0 µL	6.0 µL	60	3528 µL	72 µL
6	352.8 µL	7.2 µL	70	4116 µL	84 µL
7	411.6 µL	8.4 µL	80	4704 µL	96 µL
8	470.4 µL	9.6 µL	90	5292 µL	108 µL
9	529.2 µL	10.8 µL	100	5880 µL	120 µL
10	588.0 µL	12.0 µL			

7. Measuring procedure

1. Prepare phosphate detection reagent and enzyme master mix.
   - Note: Store enzyme master mix on ice.

2. Dilute your sample in laboratory-grade water to 5 to 200 µM polyP (as monomer).
   - Note: If the polyP concentration is unknown, test several dilutions.

3. Add 100 µL **negative control** and 100 µL of the **P_i standards** to individual wells on the microtiter plate.
   - Note: Take Table 3 as an example for a plate layout.

4. Add 100 µL of each **sample** into two different wells. Do the same with the **positive control**.
   - Note: Each sample and positive control requires two wells: one for the actual measurement and one for the blank measurement that detects contaminating phosphate.

5. Add 50 µL enzyme reaction buffer (contains no enzyme) to the P_i standards, one positive control, and the blank measurements.

6. Add 50 µL enzyme master mix to the negative control, the other positive control and the samples.

7. Mix thoroughly by pipetting 3 times up and down (avoid bubble formation).
   - Note: Set the mixing volume to 90 µl if you have an electric pipette.

8. Incubate at room temperature for 1 h.

9. Add 50 µL of the phosphate detection reagent to every well and mix by pipetting 3 times up and down.
   - Note: Set the mixing volume to 140 µl if you have an electric pipette.

10. Incubate at **room temperature for 2 min**. Remove air bubbles in the wells with fresh pipette tips.
    - Note: A longer incubation is also possible but will lead to artificially high blank readings due to non-enzymatic hydrolysis of acid -labile phosphate containing substances (e.g., polyP) in the blank.

11. Read absorption at **882 nm wavelength** in a spectrophotometric plate reader without the plate lid.

12. Calculate the sample polyP concentration using the P_i calibration curve.

Table 3: Example pipetting scheme

	Standards	Pi blanks		Negative control	PolyP measurements	
		Positive control	Sample(s)		Positive control	Sample(s)
Well	Well 1-5	Well 6	Well 7 [a]	Well 8	Well 9	Well 10 [a]
Pipetting scheme	100 µl of respective reagent					
	+ 50 µl buffer without enzyme			+ 50 µl buffer with enzyme		
	+ 50 µl Pi detection reagent					
Explanation	Calibration curve	Pi contamination…		Shows the Pi plus polyP contamination of the system	Pi plus polyP concentration…	
		of the positive control	in the sample		of the positive control	in the sample

[a] increase number of wells appropriately for multiple samples.

8. Data analysis

Either follow the instructions below or download the free Excel analysis sheet from www.aminoverse.com/Phosfinity .

Setting up a calibration curve:

Plot the measured absorption values of the P_i-standards (Y-axis) to their known concentration (X-axis; 20, 65, 110, 155, and 200 µM P_i). Draw a linear trendline through the measuring points. Do not include the negative control in the calibration curve. The formula of the calibration curve (i.e., the trendline) should be in the following form (Eq. 1).

Equation 1: $A = S * C + I$

A: sample absorption
I: trendline intersection with Y-axis
S: trendline slope
C: sample concentration [µM]

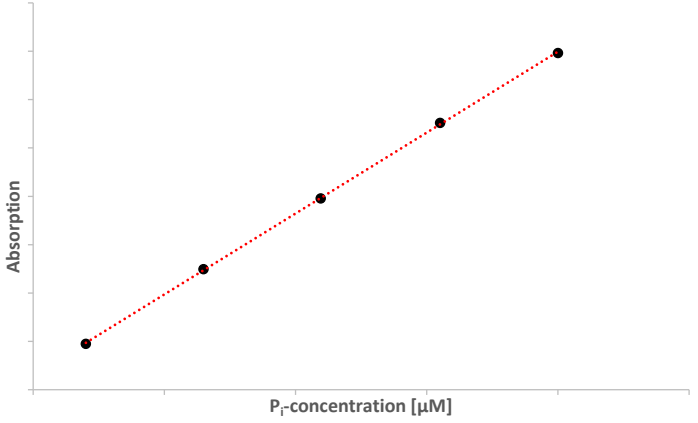

Figure 2: example standard curve

Calculating the diluted sample concentration

To convert the absorbance values to concentration values, solve the formula of the calibration curve for the variable "C" (Eq. 2). PolyP is detected as its monomer, phosphate, here. The polyP concentration is reported as µM monomer. Calculate the polyP concentration of the positive control, the samples, and the blank measurements. Only values between 5 and 200 µM polyP are valid. Repeat the measurement with higher or lower dilutions if the values are out of the linear range.

Equation 2: $$C = \frac{A-I}{S}$$

Calculation of the undiluted sample concentration

Subtract the blank value from the corresponding calculated sample polyP concentration and multiply with the sample dilution factor and multiply with the sample dilution factor (Equation 3).

Equation 3: $$true\ undiluted\ total\ polyP = (sample\ polyP - blank\ sample) * dilution\ factor$$

Quality control

The provided positive control acts as a confirmation of the kit's functionality. The acceptable range for the measured value is 80 – 120 µM polyP.

9. Interfering substances

The following substances do not interfere with the assay:

- ATP up to 2 000 µM
- ADP up to 300 µM
- Glucose 6-phosphate up to 2 000 µM
- Fructose-1,6-diphosphate up to 2 000 µM
- Phosphoenolpyruvate up to 2 000 µM
- NaCl up to 750 000 µM
- $NaSO_4$ up to 1 000 µM
- Up to 4.2 g DNA per g polyP (ca. 200 µM DNA monomers)
- Up to 4.4 g RNA per g polyP (ca. 200 µM RNA monomers)

Contaminating phosphate is detected in the blank measurement. Low polyP concentrations in comparison to the phosphate background level (e.g., < 5 µM polyP per 100 µM phosphate) might be analytically difficult to differentiate from the blank.

10. Choosing an appropriate extraction protocol

PolyP is often found as an intracellular compound, which needs to be extracted from the cell mass prior to polyP quantification. Even freely dissolved polyP might contain quantification-inhibiting substances (e.g., polyP-degrading enzymes, nucleic acids), which should be removed prior to the polyP analysis.

Three extraction procedures are recommended in combination with the kit (Table 4). However, other extraction procedures are

oftentimes similar to the here presented ones and might be suitable as well.

	Christ protocol[4]	**Bru protocol**[5]	**Werner protocol**[6]
Cell lysis	Phenol-chloroform-extraction	Phenol-chloroform-extraction	Inorganic acid (e. g., H_2SO_4)
PolyP purification	None	- DNase/RNase treatment - polyP precipitation with organic solvent	Spin column
Removal of contaminants?	No	Yes	Yes
PolyP recovery	100 %	ca. 60 %	ca. 5 %
PolyP chain length recovered	All chain lengths	Unknown, probably no short chain polyP	> 60 – 80 P-subunits
Suitable for	Cells with > 1 % polyP (as KPO_3) content in cell dry matter	All sample types	All sample types

Table 4: Comparison of analytical polyp extractions

Cells must be lysed to release the polyP. This is achieved with phenol in the Christ and Bru protocols. Phenol must be removed with chloroform later on, but does not degrade polyP. The Werner protocol uses an inorganic acid which can be easily neutralized in higher throughput. Inorganic acid, however, does not lyse the cells fully and hydrolyses polyP.

After polyP release from the cell, the Christ protocol does not further purify the polyP. Therefore, no polyP is lost. All polyP chain lengths are recovered. The polyP purity is low. In the Bru protocol, nucleic acids are degraded with DNase and RNase and the polyP is precipitated with an organic solvent (e.g., ethanol). Ca. 40 %, including short-chain polyP, of the polyP is lost during the polyP purification. In the Werner protocol, the polyP is bound to a spin column membrane. In this purification, ca. 95 % of the polyP, including polyP with a chain length shorter than 60 to 80 P-subunits, is lost. One advantage of the Bru and Werner protocols is the high polyP purity that is achieved. PolyP-degrading enzymes and other contaminants, such as magnesium, are efficiently removed. The low polyP recovery of the Werner protocol is compensated for by a convenient and quick to perform lab protocol. Concluding, there is a negative correlation between the polyP recovery and polyP purity for polyP extraction protocols. In doubt, the Bru protocol should be tested first. If you are expecting little contaminants or a cellular polyP content of >1 % polyP (as KPO_3) in dry matter, the Christ protocol might be more appropriate because it recovers more polyP.

11. Trouble shooting

Q.: *"The polyP concentration I measure is way below the expected level."*

A.: PolyP-degrading enzymes in your sample or harsh conditions (high temperature, non-neutral pH, higher valent cations, such as magnesium and calcium) might cause polyP degradation. Try a different sample extraction protocols (see the chapter above).

Is your positive control in the range of 80 to 120 µM polyp? If not, there might be an error in assay execution.

Despite its less acidic buffer system compared to competing products, the phosphate detection reagent is still able to hydrolyse acid-sensitive phosphate compounds, such as polyP, after extended periods of time. This can cause an increased blank value and will consequently lead to a falsely low estimation of the sample polyP concentration. Adhere to the 2 min incubation time after phosphate detection reagent addition before reading the absorption.

Q.: "*Is the kit suitable for mammalian cells?"*

A.: Yes. The kit measures the total concentration of dissolved polyP. Therefore, it does not matter from where the polyP stems. Two interfering substances might be faced: A) If the sample contains too much phosphate, the blank reading will be high. If the polyP concentration is low, it might be hard to differentiate the polyP from the background phosphate. B) If you have more than 4.2 g of nucleic acid per g polyP, the nucleic acid will interfere with the measurement. Use an

appropriate extraction protocol in both cases (see the chapter above).

Q.: *"I want to measure samples with a predicted high polyphosphate concentration. Why is the highest provided standard at 200 µM?"*

A.: The detection reagent has a linear range between 5 and 200 µM polyP. Every sample with a concentration above this value needs to be diluted prior to testing.

Q.: *"My sample contains a lot of nucleic acid. Can I use the kit?"*

A.: Up to 4.2 g DNA per g polyP or 4.4 g RNA per g polyP (ca. 200 µM nucleic acid monomer) are tolerated by the assay. Higher nucleic acid concentrations must be removed by an appropriate extraction procedure (see the chapter above).

Q.: *"Can I use freeze-thawed / dried cells?"*

A.: Yes. However, the polyP needs to be extracted from the cells.

Q.: *"Will the test measure both free and bound polyP?"*

A.: The kit measures all water-soluble polyP that is accessible at least from one end of the polyP chain. Covalent attachment of one polyP end is no problem.

Q.: *"I measure a few samples every day. Why isn't there enough enzyme for the stated 100 samples?"*

A.: The kit procedure includes two controls that also require enzyme. Measuring only a few samples at a time will increase the amount of enzyme used for the controls.

12. Terms and conditions

The Phosfinity kit and its components are intended for research use only, except anything else is stated in an official Aminoverse B.V. document. Furthermore, the Phosfinity kit is suitable for *in vitro* use only and has to be handled by qualified personnel.

The purchase of this product only authorises the buyer to application of the kit for internal research and does not include the right to resell, repackage or sublicense it. The buyer may also refrain from disassembling, modifying, enhancing, reverse engineering, supplementing or changing the Phosfinity kit or its components, unless stated otherwise. Furthermore, any type of clinical diagnostic or application on the human body is prohibited.

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13. References

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14. Notes

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