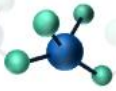
ChainQuant

Phosfinity

Polyphosphate Chain Length Determination Kit



AMINOVERSE

EXPLORING ENZYME INNOVATION

Contents

Thank you for your trust and confidence in us and our

Phosfinity ChainQuant

Polyphosphate chain length determination Kit.

We always strive to further improve our products. If you want to give feedback feel free to contact us at phosfinity@aminoverse.com.

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.

1 Introduction

Phosfinity ChainQuant provides the first commercially available option to determinate polyphosphate chain length, while remaining cost and time efficient, compared to other state of the art approaches. It applies an enzyme-based protocol and fluorescence detection, which enables high throughput screening.

Imagine a sample containing three polyP chains with a length of 8, 6, and 3 P-subunits, respectively (Figure 1). If one depicted monomer represents 1 mol polyP and the three chains are dissolved in 1 liter, the total polyP concentration is 17 mol polyP (as monomer) per liter. The chain concentration (i.e., number of polyP chains per liter) is 3 moles polyP chains per liter. The average polyP chain length of 5.67 P-subunits can be calculated by dividing the total polyP concentration by the chain concentration (i.e., 17/3). **Phosfinity ChainQuant contains two micro titer plate assays**: One for the quantification of the total polyP concentration (Phosfinity – Total PolyP Quant) and one for the measurement of the polyP chain concentration (Phosfinity – ChainQuant). Both are enzyme based and highly polyP specific.

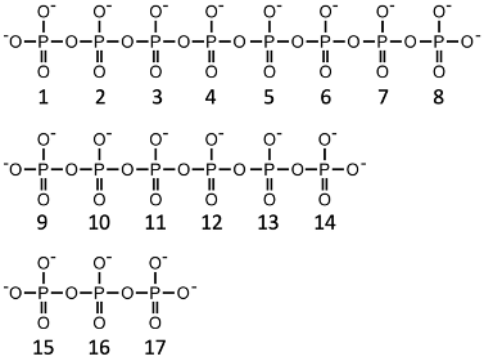


Figure 1: The principle behind Phosfinity ChainQuant

2 Key features

- Two easy to apply enzyme-based assays
- Most accurate polyP and chain length determination
- Fluorescence and absorbance based
- Affordable cost per sample
- High throughput

3 Kit components

3.1 Total polyphosphate quantification

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

3.2 Chain length concentration determination

Polyphosphate standard (1, 2.5, 5, 7.5, 10 µM)	1	mL
Negative control	1	mL
Positive control	1	mL
Enzyme B	120	µL

Enzyme C	120	µL
Enzyme additive	1	mL
Enzyme B/C reaction buffer	10	mL

4 Equipment required but not provided

- Clear flat bottom 96 well plate
- Black flat bottom 96 well plate for fluorescence
- Optional: (Multi-channel) Micro pipette
- Spectrophotometric multiwell plate reader capable of reading absorbance at 882 nm
- Fluorescence module capable of measuring at 340 nm excitation and 460 nm emission

5 Storage and stability

The **enzymes** need to be stored at **-20 °C** (avoid many freeze/thaw cycles). Transport the enzymes on ice.

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

6 Total polyphosphate quantification

6.1 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 A reaction buffer** and 1 part of the **Enzyme A** (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 A master mix

Wells	Buffer	Enzyme	Wells	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			

6.2 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 A reaction buffer (contains no enzyme) to the P_i standards, one positive control, and the blank measurements.

6. Add 50 µL enzyme A 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.
   - Note : Can be prolonged for at least 10 min.

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.

Table 3: Example pipetting scheme total polyP concentration

	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

6.3 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]

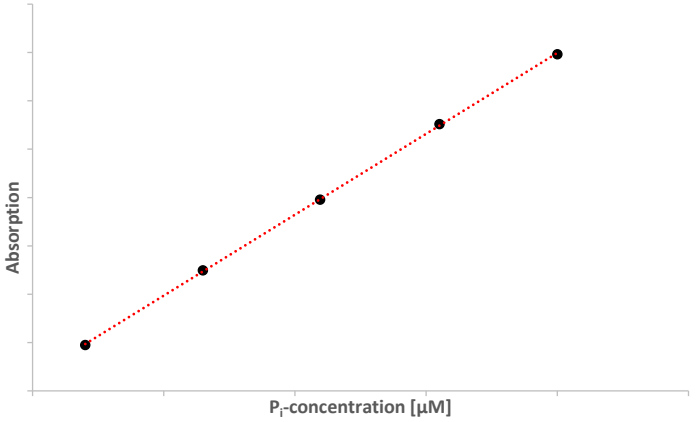


Figure 2: Example calibration curve

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:

$$C_{total\ polyP} = (C_{sample} - C_{blank}) * dilution\ factor$$

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.

7 Chain length determination

7.1 Preparation of reagents

Sample detection reagent (always prepare fresh)

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

Combine 44 µL **enzyme B/C-buffer**, 5 µL **enzyme additive** and 1 µL **Enzyme B.**

Blank detection solution (always prepare fresh)

(50 µL per well, only includes samples):

Combine 44 µL **enzyme B/C-buffer**, 5 µL **enzyme additive** and 1 µL **Enzyme C.**

Table 4: Preparing the sample detection reagent and the blank detection reagent

Wells	B/C-buffer	Enzyme additive	Enzyme B or C [a]	Wells	B/C-buffer	Enzyme additive	Enzyme B or C [a]
1	52.8 µl	6.0 µl	1.2 µl	20	1056 µl	120 µl	24 µl
2	105.6 µl	12.0 µl	2.4 µl	30	1584 µl	180 µl	36 µl
3	158.4 µl	18.0 µl	3.6 µl	40	2112 µl	240 µl	48 µl
4	211.2 µl	24.0 µl	4.8 µl	50	2640 µl	300 µl	60 µl
5	264.0 µl	30.0 µl	6.0 µl	60	3168 µl	360 µl	72 µl
6	316.8 µl	36.0 µl	7.2 µl	70	3696 µl	420 µl	84 µl
7	369.6 µl	42.0 µl	8.4 µl	80	4224 µl	480 µl	96 µl
8	422.4 µl	48.0 µl	9.6 µl	90	4752 µl	540 µl	108 µl
9	475.2 µl	54.0 µl	10.8 µl	100	5280 µl	600 µl	120 µl
10	528.0 µl	60.0 µl	12.0 µl				

[a] Use enzyme B for the sample detection reagent and enzyme c for the blank detection reagent.

7.2 Measuring procedure

If available set your fluorescence reader to room temperature (22°C)

1. Prepare the blank and sample detection solutions as described above.

   Note: A preparation of 20% excess is recommended to compensate for volume loss while using multichannel systems

2. Dilute samples in lab grade water.

   Note: If the total polyP concentration of the sample (e.g., 200 µM) and the approximate polyP chain length (e.g., 10 P-subunits) are known, use the following formula for dilution (e.g., 0.4 * 200 * 1/ 10 = 8-fold dilution).

$$dilution\ factor = (0.4 * T * \frac{1}{L})$$

T: total polyP concentration in µM polyP monomer

L: average polyP chain length in P-subunits

If both parameters are unknown, test several dilutions.

3. Pipette 100 µl of the negative control, positive control, and the five $polyP_2$ standards in separate wells of a black, 96-well, F-bottom microtiter plate.

4. Pipette 100 µl of your sample(s) each, in <u>two</u> separate wells (sample measurement and blank measurement).

5. Add 50 µl of the blank detection solution to one of the two sample wells.

6. Add 50 µl of the sample detection solution to the second of the two sample wells, the polyP$_2$ standards, the positive control, and the negative control.

7. Incubate the plate for 55 min at room temperature. Keep away from light.

   Note: The incubation time can be increased by at least 10 min.

8. Insert the plate into the plate reader for 5-10 min.

   Note: This step ensures that the plate and the plate reader have the same temperature which is critical for fluorescence reading in some plate readers. Ideally, this incubation step is combined with the fluorescence reading in one reader program to avoid unnecessary ejection of the plate between both steps.

9. Read the fluorescence (excitation 340 nm, emission 460 nm)

   Note: If available, a bandwidth of 9 nm can be chosen. If available, use the automatic gain adjustment function and scale to the 10 µM polyP standard well with a maximum scale value.

Table 5: Example pipetting scheme polyP chain length determination

Reagent	Sample(s)	Negative control	PolyP standards	Positive control	Sample(s)
Well	Well 1 [a]	Well 2	Wells 3-7	Well 8	Well 9 [a]
Pipetting scheme	100 µl of respective reagent				
	+ 50 µl blank detection solution	+ 50 µl sample detection solution			
Explanation	Contamination in the sample(s)	Negative control	Calibration curve chain concentration	Positive control	Chain concentration of sample(s)

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

7.3 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 fluorescence values of the polyP$_2$-standards (Y-axis) to their known concentration (X-axis; 1, 2.5, 5, 7.5 and 10 µM polyP$_2$). Draw a linear trendline through the measuring points. Do not include the negative control in the calibration curve.

Calculating the sample concentration

To convert the fluorescence values to concentration values, solve the formula of the calibration curve for the variable "C" (Eq. 4). Calculate the polyP concentration of the positive control, the samples, and the blank measurements. Only values between

1 and 10 µM polyP are valid. Repeat the measurement with higher or lower dilutions if the values are out of the linear range. The positive control should be 5 (± 2) µM and the negative control <1 µM $polyP_2$.

Equation 4: $$C = \frac{F-I}{S}$$

F: Fluorescence
C: PolyP chain concentration in µM
S: Slope
I: Y-achsis intercept

Calculating the average polyP chain length

Subtract the blank value from the sample and divide by 2 to receive the polyP chain concentration in µM polyP chains (Eq. 5). Subsequently, the average polyP chain length is calculated by dividing the results from **total polyphosphate quantification** and **polyP chain concentration** (Eq. 6):

Equation 5: $$C_{polyP\ chain} = \frac{(C_{sample} - C_{blank})}{2}$$

Equation 6: $$\bar{X}L = \frac{C_{total\ polyP in\ \mu M}}{C_{polyP\ chain\ in\ \mu M}}$$

C: Concentration
F: Fluorescence
S: Slope
$\bar{X}$L: Average chain length

8 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 400 000 µM
- $NaSO_4$ at least 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.

9 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.

Table 6: Comparison of analytical polyP extractions

	Christ protocol[1]	**Bru protocol**[2]	**Pohkrel protocol**[3]
Cell lysis	Phenol-chloroform-extraction	Phenol-chloroform-extraction	Chaotrophic salt (Guanidinium-thiocyanate) + heat
PolyP purification	None	- DNase/RNase treatment - polyP precipitation with organic solvent	Spin column
Removal of contaminants?	No	Yes	Yes
PolyP recovery	100 %	ca. 60 %	less than Bru protocol
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

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 Pohkrel protocol uses a chaotrophic salt in combination with heat.

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) after phenol chloroform cell lysis. Ca. 40 %, including short-chain

polyP, of the polyP is lost during the polyP purification. In the Pohkrel protocol, the polyP is bound to a spin column membrane. In this purification, a lot of the polyP, including polyP with a chain length shorter than 60 to 80 P-subunits, is lost. One advantage of the Bru and Pohkrel 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 Pohkrel 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 >0.1 % polyP (as KPO_3) in dry cell mass, the Christ protocol might be more appropriate because it recovers more polyP.

10 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.

Q.: "I detect a very high blank signal during the chain concentration assay. What could be the reason and how do I prevent this?"

A.: The reason might be a contaminations that are detected, as well. You might use an appropriate purification procedure that removes small molecules from your samples (see chapter above)."

Q.: "The average polyP chain length from the Phosfinity ChainQuant kit does not agree with my polyacrylamide gel electrophoresis result. Why is that?"

A.: The average polyP chain length can only be estimated from polyacrylamide gel electrophoresis experiments. Furthermore, polyP shorter than 15 P-subunits is not detected by polyacrylamide gel electrophoresis. The Phosfinity Chain Quant Kit measure the average polyP chain length of all polyP lengths accurately.

11 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.

Aminoverse B.V. only warrants the conformity of its products to the stated specifications and performance ranges. Aminoverse B.V. is not liable for any damages resulting from improper applications, test environments or storage conditions. Furthermore, no claims for any damages, whether direct or indirect, incidental, compensatory, foreseeable, consequential, whether based upon warranty, contract or strict liability arising in connection with the sale or the failure of the Phosfinity kit or its components to perform in accordance with the stated specifications.

Aminoverse B.V.'s sole obligation and the customer's sole remedy is limited to replacement of products free of charge in the event the kit fails to perform as warranted.

Aminoverse B.V. is not liable for damages or defects arising in shipping and handling, or out of accident or improper or abnormal use of this product; defects in products or components not manufactured by Aminoverse B.V., or damages resulting from such non-Aminoverse B.V. components or products.

Aminoverse B.V. makes no other warranty of any kind whatsoever, and specifically disclaims and excludes all other warranties of any kind or nature whatsoever, directly or indirectly, express or implied, including, without limitation, as to the suitability, reproductivity, durability, fitness for a particular purpose or use, merchantability, condition, or any other matter with respect to Aminoverse B.V. products.

12 References

1. Christ JJ, Blank LM. Analytical polyphosphate extraction from Saccharomyces cerevisiae. *Anal Biochem*. 2018;563:71-78. doi:10.1016/j.ab.2018.09.021

2. Bru S, Jiménez J, Canadell D, Ariño J, Clotet J. Improvement of biochemical methods of polyP quantification. *Microb Cell*. 2017;4(1):6-15. doi:10.15698/mic2017.01.551

3. Pokhrel A, Lingo JC, Wolschendorf F, Gray MJ. Assaying for inorganic polyphosphate in bacteria. *J Vis Exp*. 2019;2019(143):4-11. doi:10.3791/58818

13 Notes

Aminoverse B.V.
Daelderweg 9
Nuth, 6361HK
Tel.: +31 452 084815
Mail: phosfinity@aminoverse.com
www.aminoverse.com