PUB-Assay
High Phosphate Detection Kit

500 Assays 96-well plate

100 Assays 1ml cuvettes

Cat-No. CD_PI_0001

Handbook 2022-01H

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1 Introduction

Inorganic phosphate content of a sample is of importance in many industrial and scientific areas, be it food processing and quality control, environmental surveying, or process characterization in (bio)chemical or biotechnological contexts.

The PUB assay allows for the determination of fixed phosphate concentrations, but also for an online monitoring of phosphate production or consumption e.g. in enzymatic reactions.

2 Principle

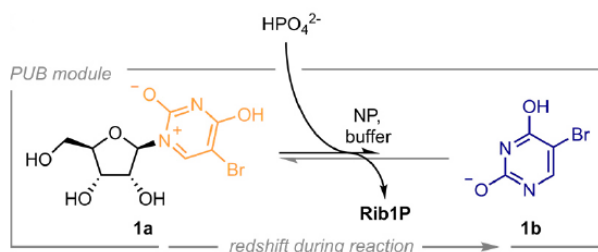

Fig. 1. Phosphate detection by continuous UV-spectroscopic monitoring of 5-bromouridine phosphorolysis (PUB module).1a: 5-bromouridine, 1b: 5-bromouracil, NP: Nucleoside phosphorylase. Graph from: [1].

Pyrimidine nucleoside phosphorylases catalyse the reversible phosphorolytic cleavage of a nucleoside to the respective base and a sugar-1-phosphate in the presence of inorganic phosphate. As the equilibrium of this reaction is subject to thermodynamic control, the initial phosphate concentration in the reaction can be obtained by determining the equilibrium conversion. For conversion calculation, the spectral differences between 5-bromouridine and 5-bromouracil are exploited using two wavelengths: At 304 nm the difference is maximal for 5-bromouridine and 5-bromouracil; the absorption at 288 nm is used for normalization as absorbance is the same for both species (isosbestic point).

During the reaction, the absorption at 304 nm will increase over time and stagnate at a certain level, depending on phosphate concentration. Once a constant value is reached, monitoring can be stopped, and the last obtained values (equilibrium conversion) are used to calculate the phosphate concentration in the reaction. In case of at line monitoring of the reaction, multiple time points should be taken to ensure that the equilibrium was reached.

3 Sensitivity and Precision

The assay enables analysis of inorganic phosphate content of a sample in the range of >75 µM to 10 mM with reasonable accuracy.

4 Safety

Please adhere to all safety measures that generally apply when handling chemical substances and refer to the supplied MSDS.

5 Kit contents and Storage conditions

Component	Storage	Comment
5-Bromouridine	-20°C (both powder and stock solution)	It is recommended to aliquot before first use.
Reaction buffer	+4°C (short term storage)	It is recommended to aliquot before first use and store at -20°C.
Nucleoside Phosphorylase Y02	-20°C	It is recommended to aliquot before first use.
5-Bromouracil standard	-20°C	

5-Bromouridine is supplied as powder (stable >1 year when stored below -10°C). After addition of 2 mL ultrapure water, the concentration is 10 mM. To accelerate complete solving, the solution may be shaken vigorously, subjected to ultrasound for 10 min or heated to 40°C for 10min. The solution is stable for 10 days at +4°C. For long term storage, it is advisable to prepare aliquots and store the stock solution at -20°C. Avoid repeated freeze-thaw cycles.

The supplied buffer is sterile filtered but contains no conservative. It is stable for 4 weeks at +4°C. To prevent microbial growth in the buffer, preparation of aliquots and storage at -20°C is recommended.

The enzyme is provided in a 50% Glycerol solution to enable long term stability upon cold storage (>1 year when stored below -10°C. Storage at -20°C is recommended).

6 Recommended Equipment

- Any spectrophotometric device able to measure absorbance at 288 nm and 304 nm is suitable. Examples are spectrophotometers for cuvettes, micro well plate readers and micro volume spectrophotometers. The cuvettes or micro well plates must be UV-compatible.
- Plastic reaction tubes for master mix preparation.
- Variable volume micropipettes (200 µL and 1000 µL)

7 Procedure

1. The preparation of a blank is necessary to be able to subtract the background signal from the sample measurements. The blank is prepared with buffer and the phosphate containing sample in the same concentration as in the reaction.

2. The enzyme is sensitive towards regular thawing and freezing. Therefore, it should only be removed from -20°C as short as possible. The enzyme should be on ice or in a cooled transport rack when being outside of the -20°C freezer.

3. Prepare your sample to fit the described phosphate concentration in the reaction. Prepare the reaction mixture without your phosphate sample. Add the phosphate sample and incubate for up to 20 min to allow reaction to reach equilibrium. If in doubt, measure again after another 5min to ensure that the reaction is finished.

Table 1. Reaction mix for 200 µl reactions.

Component	**Stock concentration**	**Final concentration**	**200 µl reactions**	**Blank**
5-Bromouridine	10 mM	0.15 µM	3 µl	-
Reaction buffer	800 mM	200 mM	50 µl	50 µl
Nucleoside Phosphorylase Y02	2 mg/ml	40 µg/ml	4 µl	-
			Ad 200 µl	Ad 200 µl
Sample*			≤143 µl	≤143 µl

* The final phosphate concentration should be in the range of 75 µM to 10 mM.

Table 2. Reaction mix for 1 ml reactions.

Component	**Stock concentration**	**Final concentration**	**1 ml reactions**	**Blank**
5-Bromouridine	10 mM	0.15 µM	15 µl	-
Reaction buffer	800 mM	200 mM	250 µl	250 µl
Nucleoside Phosphorylase Y02	2 mg/ml	40 µg/ml	20 µl	-
			Ad 1 ml	Ad 1 ml
Sample*			≤715 µl	≤715 µl

* The final phosphate concentration should be in the range of 75 µM to 10 mM.

8 Calculation

The calculation of the phosphate concentration in your sample can be done as described in the following paragraphs, or with the help of an excel sheet available at www.bionukleo.com/downloads/.

First, the 288 nm and 304 nm signal of the blank have to be subtracted from the respective signals of the reaction. Then, the ratio of the 304 nm to the 288 nm signal is calculated. The conversion of 5-bromouridine to 5-bromouracil is calculated as follows:

$$c_{eq} = \frac{A_{\frac{304}{288},sample} - A_{\frac{304}{288},BrUrd}}{A_{\frac{304}{288},BrUra} - A_{\frac{304}{288},BrUrd}}$$

where c is the conversion from 5-bromouridine to 5-bromouracil in a decimal number, $A_{304/288,sample}$ is the ratio of the observed absorbances at 304 nm and 288 nm, and $A_{304/288,BrUrd}$ and $A_{304/288,BrUra}$ the respective values of the nucleoside and the base.

With the supplied buffer, the formula is:

$$c_{eq} = \frac{A_{\frac{304}{288},sample} - 0.154}{0.973 - 0.154}$$

although the parameters might differ slightly in your system.

The phosphate concentration in your sample is calculated as follows:

$$[P]_0 = \frac{(c_{eq} * 0.15)^2}{0.16 * 0.15 * (1 - c_{eq})}$$

Where $[P]_0$ is the phosphate concentration of your sample in mM, c_{eq} is the equilibrium reached in a decimal number, 0.15 is the concentration of substrate in mM, 0.16 is the equilibrium constant of 5-bromouridine phosphorolysis.

9 Troubleshooting

Observations	**Reason**	**Solution**
The conversion is negative.	System specific factors influence the measured absorbances.	Measure absorbance at 288 nm and 304 nm of a blank (see *Tab*. 1 or 2) containing either 0.15 mM 5-bromouridine (substrate) or 0.15 mM 5-bromouracil (product) and exchange the standard $A_{\frac{304}{288}}$ -values with your observed absorption ratios.
Low or no conversion is observed.	The enzyme is inhibited.	Higher dilution of the sample should be tested. Check if equilibrium changes after re-addition of enzyme. Add enzyme until equilibrium is no longer influenced.
	Sample absorbs at 288 and/or 304nm.	Measure a bank with sample in buffer and enzyme without substrate and subtract the absorptions at 288 nm and 304 nm from the absorptions in the reaction sample.
	The sample is changing reaction pH.	Adjust sample pH to 9 or dilute your sample.
Further observations.	The calculated phosphate concentration is out of range.	> 10 mM Pi: Dilute your sample. < 75 µM Pi: Use the more suitable kit for low phosphate concentrations.
	Sample contains solids.	Remove solids by centrifugation of filtration.
	Reaction turns turbid	Dilute your sample.
	Reaction is not finished.	Increase enzyme concentration or incubation time.

1. Eilert, L.; Schallmey, A.; Kaspar, F. UV-Spectroscopic Detection of (Pyro-)Phosphate with the PUB Module. Anal. Chem. 2022, 94 (8), pp. 3432–3435.

WITHOUT GUARANTEE

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