UV-Spectroscopic Detection of (Pyro-)Phosphate with the PUB module

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Despite the prevalence of ortho- and pyrophosphate in biochemistry, operationally simple and versatile high-throughput methodologies for their quantification are lacking. We herein introduce PUB, a module for phosphate detection by continuous UV-spectroscopic monitoring of 5-bromouridine phosphorolysis. The PUB module employs cheaply available, bench-stable reagents and can be employed for continuous and discontinuous reaction monitoring in biochemical assays to detect (pyro-)phosphate concentrations spanning almost four orders of magnitude, as demonstrated with representative use-cases.

Phosphate groups are ubiquitous across the biological sciences as they serve as allosteric modifiers, solubility anchors or high-energy leaving groups (Figure 1a). As such, nature has developed a myriad of enzymes to shuffle phosphate groups between substrates and/or utilize energy-rich phosphorylated cosubstrates to power otherwise unfavorable reactions (Fig. 1b). Examples of such enzymes span transferases, ligases and lyases and account for more than 10 million entries in the Uniprot database. Despite their prevalence, the experimental interrogation of many of these enzyme functions (particularly their kinetics) remains difficult since the detection of inorganic (pyro-)phosphate is not trivial in many biochemical assays. Notable previous developments toward this end include fluorescent phosphate binding proteins,[2] fluorescent ionic dyes with tunable photochemical properties,[3] chromogenic metal complexes[4] and coupled enzymatic assays employing urine nucleoside phosphorlyases and xanthine-oxidases to generate a UV signal.[5] However, these methods typically only allow for the examination of a small experimental space, exhibit low selectivity, or employ expensive reagents unsuited for high-throughput experimentation (see the Supplementary Information and Table S1 for a detailed literature analysis).

Thus, cheaply available, operationally simple, and versatile methods, which are compatible with biochemical assays and high-throughput experimentation, are lacking. To close this gap, we developed PUB, a compact module for phosphate detection via UV-spectroscopic monitoring of 5-bromouridine phosphorolysis. Herein, we introduce the PUB module, outline its application to biochemical assays on various scales and illustrate its utility with selected use-cases.

The PUB module relies on a highly selective nucleoside phosphorolysis step, which can be leveraged to generate a UV spectroscopic read-out in the presence of phosphate. Given the inherent limitations of existing purine-based systems, which employ irreversible enzymatic phosphorolysis of a nucleoside precursor, we recognized that a conceptual redesign would be necessary to realize the versatility and scope required for many applications. Based on our previous forays into nucleoside phosphorylase chemistry[6–9] and UV spectroscopic reaction monitoring,[10,11] we hypothesized that we could employ in situ deprotonation of a pyrimidine nucleoside and its corresponding nucleobase, which then tautomerizes to a pyrimidinolate, to yield a spectral redshift (Fig. 1c). Thus, we envisioned that the phosphorolysis of a pyrimidine nucleoside could report on the presence of phosphate in solution through a characteristic UV spectroscopic signal reflecting conversion of the starting material. A systematic examination of commercially available uridine analogs identified 5-bromouridine (1a) as the ideal candidate. The nucleoside 1a ($pK_a = 7.95$) can be deprotonated under mildly alkaline conditions in buffered solution and its corresponding anionic nucleobase 1b ($pK_a = 8.03$) exhibits a 22 nm UV redshift, enabling their spectroscopic differentiation. Monitoring of the phosphorolysis of 1a to 1b at pH 9 yields highly overlapping absorption spectra with an isosbestic point at 288 nm (Fig. 1d) and a maximum difference of the extinction coefficients at 304 nm ($\Delta\varepsilon_{304} = 5.4$ mM$^{-1}$ cm$^{-1}$, Figs. 1e and 1f). Thus, monitoring at 304 nm coupled with isosbestic point normalization via parallel monitoring at 288 nm provides quantitative real-time conversion data for this transformation, given reference values for both compounds (Fig. 1g).

An extensive evaluation of buffer systems revealed that the PUB module performs best in amino acid buffers such as aspartic acid ($pK_a = 8.8$) or glycine ($pK_a = 9.6$) or the amino sulfonate taurine ($pK_a = 9.1$). Sulfonic acids from Good’s series[12] and the “better buffer” diethylpiperazine[13] gave inferior results while other buffers including ammonia, borate or carbonate proved incompatible (see the SI for a detailed analysis and buffer recommendations; also see the SI section “Additional considerations”). Owing to the inherent selectivity of nucleoside phosphorylases, the PUB module is highly selective to inorganic phosphate and exhibits no cross-reactivity with other phosphate species. Similarly, we found that the presence of other ions in solution has no significant impact on the performance of the module.

The optimized PUB module is a highly robust system capable of detecting (pyro-)phosphate over a remarkable concentration range by exploiting principles of thermodynamic control. While previous nucleoside phosphorylase-based systems have used irreversible phosphorolytic steps (either via product removal or by rendering the reverse direction kinetically unfeasible), the PUB module employs a reversible phosphorolysis, which yields a significantly simplified system in terms of required materials. As such, the PUB module utilizes the tight thermodynamic control of the phosphorolysis of 1a ($K = 0.16$) to detect multiple equivalents of phosphate per nucleoside (Fig. 1h). Using modifications of previously published equations,[11] the extent of conversion in an equilibrium state of this transformation (as monitored spectroscopically via the $A_{304}/A_{288}$ ratio) provides the phosphate concentration in solution with high accuracy and reproducibility. In practice, this means that 100 µM to >5 mM phosphate can readily be detected with standard spectroscopic read-out in the presence of phosphate.
The versatility of phosphate in nature is driven by a vast array of phosphate-shuffling enzymes.

**Figure 1.**

**a** Examples of phosphorylated biomolecules. **b** Phosphate turnover in vivo requires enzyme for phosphate shuffling.

The PUB module enables quantitative phosphate detection in biochemical assays.

**c** PUB module

**d-e** UV absorption spectra obtained during phosphorolysis of 1a at pH 9.

**f** pH-dependence of the spectroscopic properties.

**g** Continuous reaction monitoring of the 1a → 1b transformation via isosbestic point normalization.

**h** Thermodynamic control of the phosphorolysis of 1a enables the detection of multiple equivalents of phosphate in various amino acid buffer systems.

**i** Extinction differences enable detection of low phosphate concentrations.

**j** Non-linear response of ΔA to phosphate due to thermodynamic control.

**k** Phosphate can be detected accurately over concentrations spanning more than three orders of magnitude.

**l** Enzymatic hydrolysis of pyrophosphate to orthophosphate.

**m-o** Detection of pyrophosphate via thermodynamic control following hydrolysis.

**p** Thermal stability of the IPP from *Geobacillus stearothermophilus*.

**q** Eyring plots of IPP-catalyzed pyrophosphate hydrolysis, revealing curvature due to an activation heat capacity change.

**r** Phosphatase-catalyzed hydrolysis of organophosphates, Michaelis-Menten kinetics with selected model substrates obtained via high-throughput experimentation.

PUB = phosphate detection via UV spectroscopic monitoring of 5-bromouridine phosphorolysis, NB = nucleobase, NP = nucleoside phosphorylase, Rib1P = ribose-1-phosphate, eq. = equivalent, IPP = inorganic pyrophosphatase, DMSO = dimethylsulfoxide, K = equilibrium constant. All data are freely available online.\(^\text{[14]}\)
scopic equipment, with continuous and discontinuous monitoring approaches giving essentially identical results in a high-throughput setup. Notably, this approach can be carried out in 1 mL cuvettes in a photometer, 200 µL multiwell plates in a plate reader or even in 1 µL droplets assessed with a nanophotometer. Lower phosphate concentrations down to around 2 µM can be detected by providing 1a in excess and measuring absorbance differences between a baseline and a near-equilibrium state (Figs. 1i and 1j). The latter can be fitted with first-order kinetic assumptions for an accurate assessment of phosphate content up to at least 200 µM (see the SI for details, equations and workflow). Collectively, this enables the quantification of phosphate over a concentration range spanning nearly four orders of magnitude (Fig. 1k). While we recommend analysis at pH 9 for optimal performance, the PUB module can also be employed at lower pH values, albeit with reduced sensitivity due to smaller ΔT values (Fig. 1f; please also see the SI section “Additional considerations”). The extension of this methodology by an inorganic pyrophosphatase (IPP, hydrolyzing pyrophosphate to orthophosphate, Fig. 1l) enables the quantification of pyrophosphate in a fully analogous fashion, employing identical reaction conditions (Figs. 1m–o).

As all these approaches are readily compatible with continuous reaction monitoring and high-throughput experimentation, the PUB module is a remarkably useful tool for kinetic studies and enzyme characterization, as we demonstrated with selected use-cases. For instance, we assessed the thermal stability of the IPP from Geo bacillus steatorrhophilus and found the T_50 value determined through kinetic experiments to be in excellent agreement with the noisy T_m obtained via differential scanning fluorimetry (Fig. 1p). As the ΔF304 between 1a and 1b is not temperature-dependent and Y02 readily allows experimentation up to at least 60 °C (see the SI for details), we used the PUB module to obtain Arrhenius plots for the IPP-catalyzed pyrophosphate hydrolysis (Fig. 1q). This analysis revealed the presence of a moderate activation heat capacity change ΔC_p across different conditions, [6] which would have been impossible or extremely laborious to examine with existing phosphate detection methods. In a similar manner, the PUB module allowed the straightforward characterization of an alkaline phosphatase with a series of model substrates 2a–2d (Figs. 1r and 1s). Notably, these experiments could routinely be performed in a multi-well format running more than 30 reactions in parallel.

In conclusion, we have developed PUB, an operationally simple module for the detection of inorganic (pyro-)phosphate in biochemical assays. This method is cheap, versatile, and readily compatible with standard spectroscopic equipment and high-throughput experimentation on various scales.

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Conflict of Interest
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Supplementary Information
The following references appear in the Supplementary Information:
CRediT system
Scientific color maps
Golden Gate cloning
Phosphate detection from plant samples
Phosphate detection methods
Malachite green-based assays
Other molybdate-based assays
Coumarin-based sensors
NADH-based enzymatic assays
Xanthine oxidase-based enzymatic assays
Methyl-purine-based enzymatic assays
Thymidine phosphorylase-based, HPLC-coupled assays
Thioflavine T-based fluorophores
Pyro-phosphatase-based assays
Pyruvate oxidase-based assays
Phosphate-binding proteins
Metal-binding fluorescent dyes
Colorimetric assays with insolubles
Capillary electrophoresis

Phosphorolysis reaction monitoring

Nucleoside phosphorolysis reaction monitoring

Phosphate detection methods
NMR-based detection
Zn2+-based fluorescent sensors
Cu2+-based fluorescent sensors
Cd2+-based fluorescent sensors
Ga3+-based fluorescent sensors
Fe3+-based fluorescent sensors
Al3+-based fluorescent sensors
Amide-based sensors
Carboxylate-based sensors
Imidazolium-based sensors
Pyridinium-based sensors
Pyrrrole-based sensors
Guanidinium-based sensors
(Thio-)-Urea-based sensors
Gold nanoclusters
Xanthine oxidase-based enzymatic assays
NADH-based enzymatic assays
UDP-glucose phosphorylase-based enzymatic assays
Luciferase-based assays
Electrochemical methods
Precipitation
Heterologous xanthine oxidase expression
Xanthine oxidase instability
Instability of 7-methylated purine nucleosides
Biocatalytic nucleoside synthesis
Thermostable nucleoside phosphorylases
5-Fluorouracil pKa calculation
Nucleoside phosphorylase reaction monitoring


