The AttoPhos® System for Fluorescent Detection of Alkaline Phosphatase in an Enzyme-Linked Assay

By Dan Simpson, Ph.D.[1], Bill Daily, Ph.D.[2], and Scott Brehm, B.S.[2]
[1]Promega Corporation and [2]Promega Biosciences, Inc.

Promega's new AttoPhos® System provides a fluorescence-based method for the rapid and sensitive detection of alkaline phosphatase in solution. This article discusses general principles of fluorescence-based enzyme-linked assays and highlights the characteristics of the AttoPhos® System that contribute to its sensitivity and utility.

Introduction

Many assays require the measurement of alkaline phosphatase (AP) as an indication of the presence, or for quantitation of, specific molecules (e.g., proteins and nucleic acids; 1–11). Alkaline phosphatase is also commonly used as a marker for monitoring the level of antibodies, drugs, enzymes and other analytes in a wide range of biological tests. Most of these monitoring techniques use a substrate for AP that will produce a chemiluminescent, colorimetric or fluorometric signal, providing the ability to quantitate the level of AP present in a sample.

Promega has recently introduced the AttoPhos® System for the sensitive detection of AP in solution. The system is based on the fluorometric AttoPhos® Substrate, which allows rapid detection of AP down to the attomole (10^{-18} mole) level. Fluorescence-based techniques such as the AttoPhos® System offer advantages over other methods with regard to convenience, sensitivity, speed and relative quantitation. In this article, we discuss these advantages as well as some general considerations for using fluorometric reagents for the detection of AP.

General considerations for fluorescence-based assays

As a reporter, AP functions by hydrolyzing the phosphate ester (or esters) of an exogenous substrate to the corresponding alcohol and inorganic phosphate (P_i). The reaction of the AttoPhos® Substrate with AP to produce BBT (2´-[2-benzthiazoyl]-6´-hydroxy-benzthiazole) and P_i is illustrated in Figure 1.

For colorimetric substrates (e.g., para-nitrophenyl phosphate [pNPP]), the UV-visible absorbance properties of the phosphate ester and the alcohol generally differ enough that the presence of the alcohol in solution can be distinguished solely on the basis of an absorbance measurement. The detection limit is the lowest concentration at which the alcohol can be detected in a defined concentration of the phosphate ester. For fluorometric substrates such as the AttoPhos® Substrate the absorbance change is accompanied by a change in the fluorescence emission wavelength and an increase in fluorescence quantum yield. This allows more efficient discrimination of the product alcohol from the phosphate ester and thus provides significantly higher sensitivity when applied to the detection of AP. The differences in optical properties for several common AP substrates are shown in Table 1.

In practical applications such as fluorescence-based ELISAs the endogenous background produced by other assay components in part defines the assay sensitivity. For fluorescence-based assays, this background includes the intrinsic fluorescence of any components other

Table 1. Absorption and Fluorescence Properties of AP Substrates.

AP Substrate	Absorbance max (nm)	Absorbance (ε, $M^{-1}cm^{-1}$)	Fluorescence excitation max. (nm)	Fluorescence emission max. (nm)
pNPP	311	9,800		
pNP	405			
AttoPhos®	360	29,000	410	450
BBT	418	26,484	440	560
4-MUP	320	15,000	352	390
4-MU	360	19,000	360	440
FDP	272	5,000		
Fluorescein	490	61,400	490	520

ε = extinction coefficient.
pNPP = para-nitrophenyl phosphate
BBT = 2´–[2-benzthiazoyl]-6´–hydroxy-benthiazole
4-MUP = 4–methylumbelliferyl phosphate
FDP = fluorescein diophosphate

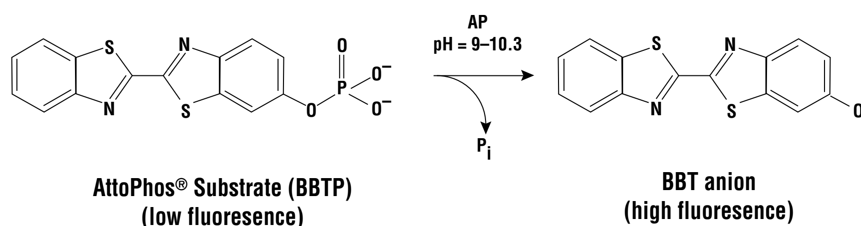


Figure 1. AttoPhos® Substrate reacts with alkaline phosphatase (AP) to produce BBT and P_i.

E-mail *dsimpson@promega.com* for technical questions

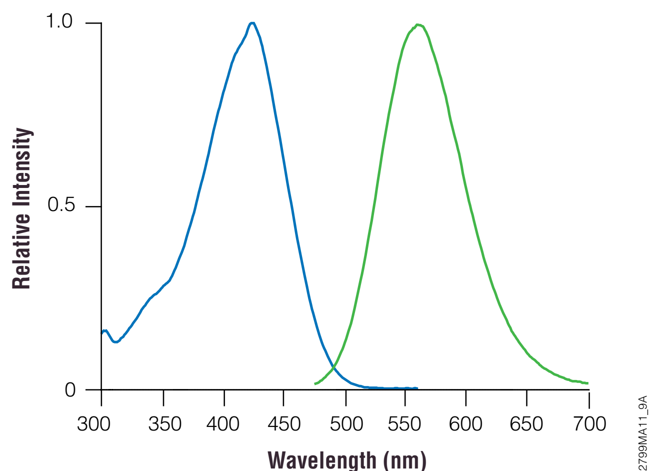


Figure 2. Fluorescence excitation and emission spectra demonstrating the large Stokes' shift for BBT, the product of the reaction of AP with AttoPhos® Substrate. The green line represents the excitation spectrum collected at an emission wavelength of 575nm. The yellow line represents the emission spectrum collected at an excitation wavelength of 440nm. Both spectra have been normalized for comparison.

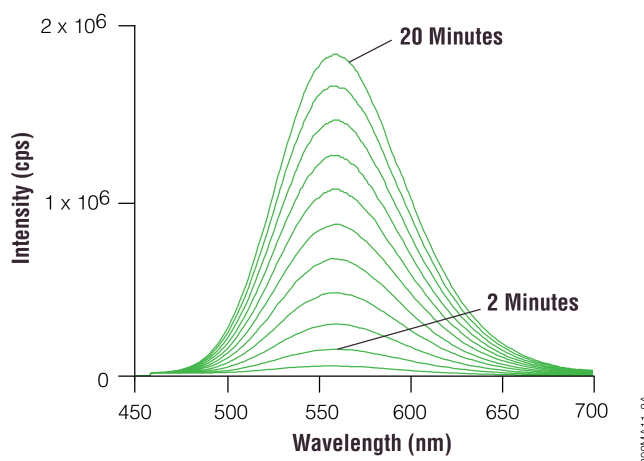


Figure 3. Fluorescence emission spectrum of BBT. Alkaline phosphatase (180pg/ml) was treated with 1mM AttoPhos® Substrate. Fluorescence emission (450nm excitation) was measured for 20 minutes at 2-minute intervals. The reaction was performed in a 3ml cuvette at ambient temperature (22–24°C) with constant stirring.

than the substrate and its product. When choosing a fluorescent reporter to apply to a detection scheme one should consider the following: i) The absorbance or excitation wavelength of the dye (or product) should be chosen to avoid interference of any endogenous background signal. (Thus, wavelengths greater than 400nm are generally preferred.) ii) A large Stokes' shift, defined as the difference in wavelength between the excitation wavelength and the emission wavelength, is desirable. iii) A high quantum yield, defined as a fluorophore's efficiency in converting excitation light into fluorescence emission, is also desirable.

In the absence of any endogenous background, the combination of Stokes' shift and quantum yield define the sensitivity of most fluorescent assays. Fluorophores with a small Stokes' shift (<50nm) require a narrower fluorescence emission window to be collected, resulting in reduced sensitivity. This decrease in emission window must be counteracted by other properties such as quantum yield. Furthermore, in solution, the excitation-dependent Raman scattering of water can contribute significantly to the background signal. This becomes more important for narrow emission windows and in cases where the excitation wavelength is less than 400nm. Fluorescent compounds with large Stokes' shifts (>50nm) and excitation wavelengths above 400nm require less stringent filtering and have a greatly reduced contribution from Raman scattering to the background signal. This allows the collection of a broader area of the fluorescence emission and results in increased relative sensitivity. Figure 2 shows the large Stokes' shift observed for BBT, the fluorescent product of the reaction of AttoPhos® Substrate with AP.

Whenever possible, the use of AP or any enzyme as a fluorescent reporter offers a significant advantage over direct fluorescent labeling approaches. Turnover of the enzyme allows for the accumulation of a large number of fluorescent molecules, resulting in amplification of the signal by log factors. An example of this is shown in Figure 3, which shows the time-dependent fluorescence emission of BBT during the reaction of AP with AttoPhos® Substrate.

Desirable properties for any enzyme-based reporter include high enzyme turnover (>50,000 molecules/minute/AP molecule), low K_m values and little to no interference or inhibition of the enzyme by the reaction product. The turnover of AttoPhos® Substrate with AP under optimized conditions is >90,000 molecules/minute/AP molecule and the K_m is 0.3mM. The combination of low K_m and high turnover allows reduced spectral background from the substrate while maintaining optimal signal generation by keeping the enzyme completely saturated with substrate during the reaction. AP is inhibited by inorganic phosphate (P_i), which is produced by all AP-substrate reactions. However, the level of P_i required to significantly inhibit the enzyme (0.28M) is well above the amount produced in a typical reaction.

Multiwell plate-based detection of AP with AttoPhos® Substrate

The AttoPhos® System provides the AttoPhos® Substrate as a dispensed powder together with an optimized buffer (AttoPhos® Buffer) for the detection of AP. A standardized solution of BBT (500ng/ml) is also provided and can be used for instrument calibration or as a standard for stoichiometric quantitation of the BBT produced in the assay. The advantages of this system include: i) reduced background fluorescence due to biological molecules that exhibit absorbance in the 340–430nm range; ii) reduced light scattering associated with solution turbidity, sample cuvette or multiwell plate construction due to the longer excitation wavelength (450nm v. 350nm); iii) a large Stokes' shift that allows the collection of a broad emission window and good compatibility with a wide range of fluorescence instrumentation.

Any fluorometer in which the excitation wavelength can be set within a range of 420–460nm and the emission wavelength greater than 525nm is well suited for use with the AttoPhos® Substrate. Sources of suitable multiwell plate instruments include BioTek, LabSystems, PerSeptive Biosystems and Dynex. For optimal results, the excitation wavelength should be set in the 430–440nm range and the emission wavelength should be set in the 550–560nm range. However, BBT has been successfully detected with wavelengths as short as 365nm and as long as 532nm.

The AttoPhos® Reagent is prepared by dissolving the AttoPhos® Substrate in AttoPhos® Buffer to yield a 1mM solution that is ready for use. Application of the substrate only requires careful pipetting to the samples of interest. Care should be taken to avoid contamination with exogenous AP when preparing the substrate and the samples to be

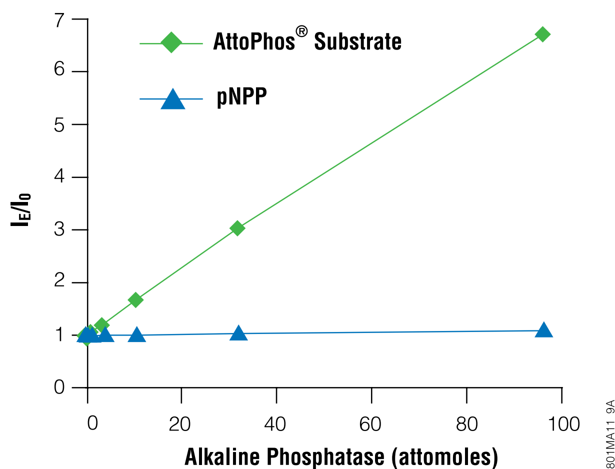


Figure 4. Fluorescence signal observed for a serial titration of AP treated with 1mM AttoPhos® Substrate in a multiwell plate. Fluorescence and OD_{405} signals observed for a 10-minute incubation of AP with AttoPhos® Substrate and pNPP. The data are presented as the ratio of the signal obtained for each enzyme amount to the signal observed for no enzyme (I_E/I_0). The AP standards were prepared by diluting stock AP (365µg/ml) in buffer containing 50mM Tris-HCl (pH 9.3), 1mM $MgCl_2$, 0.1mM $ZnCl_2$, 1mM spermidine and 0.1% BSA in a concentration range from 4fM to 26pM. The pNPP solution was prepared by diluting 10mM pNPP to 1mM in buffer containing 1M diethanolamine and 1mM $MgCl_2$. The colorimetric detection of pNPP was carried out on a Molecular Devices SpectraMAX® plate reader at 405nm. The 200µl reaction was performed in a Linbro®/Titertek® flat-bottom 96 well plate. Fluorescent detection of AP with AttoPhos® Substrate was performed in a Dynex MicroFLUOR® 96 well plate in a volume of 100µl and measured on a PerSeptive Biosystems Cytofluor® II plate reader. The filters used were optimized for the AttoPhos® System reaction products BBT: 450nm (50nm bandpass) excitation and a 580nm (50nm bandpass) emission filter. The limit of detection (0.74 and 46 attomoles for AttoPhos® Substrate and pNPP, respectively) in both cases was 2 standard deviations above the average value obtained for no enzyme.

interrogated. When analyzing samples containing low levels of AP ($\leq 10^{-14}$M), any reagents used in the assay should be checked in advance for their effect (blank rate) on the system background drift. The effect of trace quantities of metal on the spontaneous hydrolysis of AttoPhos® Substrate can be a problem when testing for very low levels of AP ($\leq 10^{-14}$M). Sources of metal ions should be maintained at the lowest practical level.

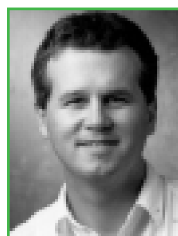
The AttoPhos® System is easily adapted for use in high-throughput multiwell plate assays. Figure 4 illustrates the advantage the AttoPhos® System offers over colorimetric assays for the detection of AP. For colorimetric detection, the AP solution was treated with pNPP. The signal observed for each enzyme amount is shown as a ratio of the signal in the absence of enzyme (I_E/I_0). The response observed was linear over the range of AP amounts detected. The limit of detection for pNPP in this assay was 46 attomoles at 10 minutes. In contrast the AttoPhos® System demonstrated a limit of detection of 0.74 attomoles or 60 times lower than that observed for pNPP over the same 10-minute period.

Summary

The AttoPhos® System provides several benefits for the rapid and sensitive detection of AP in enzyme-linked assays. These include red-shifted excitation and emission wavelength that reduce potential fluorescence background from interfering biological species, a large Stokes' shift resulting in a broad spectral window and rapid enzyme kinetics for turnover of large numbers of fluorescent molecules. The longer wavelength properties and large Stokes' shift also allow greater accuracy in turbid solutions or solutions prone to high light scattering due to particulates. The system is provided with an optimized buffer, is well suited for high-throughput multiwell plate-based assays and offers AP detection limits at or substantially below the 1 attomole level.

References

1. Parandoosh, Z., Bogowitz, C.A. and Nova, M.P. (1998) *In Vitro Cell. Dev. Biol. (Animal)* **34**, 772.
2. Hanes, J. and Pluckthun, A. (1997) *Proc. Natl. Acad. Sci. USA* **94**, 4937.
3. Craig, D.B. *et al*. (1996) *J. Amer. Chem. Soc.* **118**, 5245.
4. Craig, D.B., Wong, J.C.Y. and Dovichi, N.J. (1996) *Anal. Chem*. **68**, 697.
5. Maier, E., Crollius, H.R. and Lerach, H. (1994) *Nucl. Acids Res*. **22**, 3423.
6. Cherry, J.L. *et al*. (1994) *Genomics* **20**, 68.
7. Kerkhof, L. (1992) *Anal. Biochem.* **205**, 359.
8. Cano, R.J. *et al*. (1992) *BioTechniques* **12**, 264.
9. Cano, R.J. *et al*. (1992) *Eur. J. Clin. Micro. Inf. Dis.* **11**, 602.
10. Cano, R.J. *et al*. (1992) *J. App. Bact.* **72**, 393.
11. Wright, S.D. *et al.* (1992) *J. Exp. Med*. **176**, 719.



Dan Simpson

Bill Daily

Scott Brehm

Ordering Information

Product	Size	Cat.#
AttoPhos® AP Fluorescent Substrate System	3 × 36mg	S1000

AttoPhos is a trademark of Promega Corporation and is registered with the U.S. Patent and Trademark Office.

CytoFluor is a registered trademark of Millipore Corporation. Linbro is a registered trademark of Linbro Scientific, Inc. MicroFLUOR is a registered trademark of Dynex Technologies, Inc. SpectraMAX is a registered trademark of Molecular Devices Corporation. Titertek is a registered trademark of Flow Laboratories, Inc.