



Promega

Technical Bulletin

AttoPhos[®] AP Fluorescent Substrate System

INSTRUCTIONS FOR USE OF PRODUCTS S1000 AND S1001.



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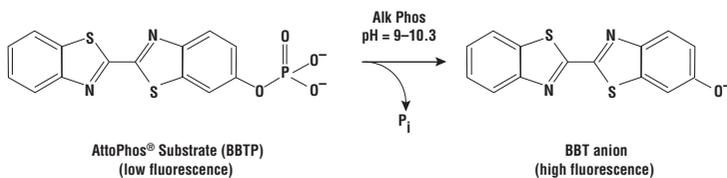
AttoPhos[®] AP Fluorescent Substrate System

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1. Description

The AttoPhos[®] AP Fluorescent Substrate System^(a) provides a highly sensitive fluorescent alkaline phosphatase (AP) substrate. The system includes AttoPhos[®] Substrate, AttoPhos[®] Buffer and AttoPhos[®] Calibration Solution. AttoPhos[®] Substrate (2'-[2-benzothiazoyl]-6'-hydroxybenzothiazole phosphate [BBTP]) is cleaved by alkaline phosphatase to produce inorganic phosphate (P_i) and the alcohol, 2'-[2-benzothiazoyl]-6'-hydroxybenzothiazole (BBT) [Figure 1].



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Figure 1. The reaction of AttoPhos[®] Substrate with AP to produce BBT and inorganic phosphate (P_i).

1. Description (continued)

The enzyme-catalyzed conversion of the phosphate form of AttoPhos® Substrate to BBT is accompanied by an enhancement in fluorescence properties. Relative to AttoPhos® Substrate, BBT has greatly increased quantum efficiency, and fluorescence excitation and emission spectra that are shifted well into the visible region. Relative to other fluorometric reporters, the BBT anion has an unusually large Stokes' shift of 120nm, which leads to lower levels of background fluorescence and higher detection sensitivity (Figure 2).

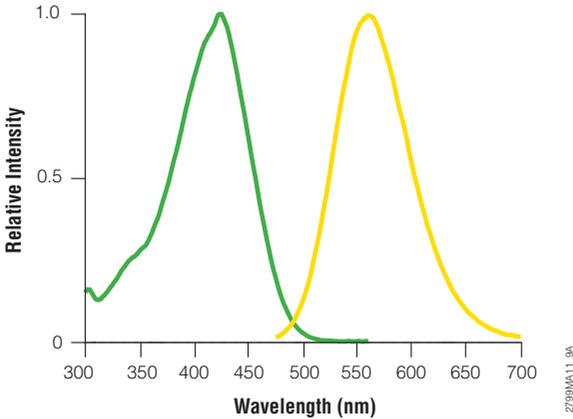


Figure 2. Fluorescence excitation and emission spectra for BBT. The excitation spectrum (max. 435nm) was collected with an emission wavelength of 575nm. The emission spectrum (max. 555nm) was collected at an excitation wavelength of 440nm. Both spectra were normalized for comparison.

2. Product Components

Product	Cat.#
AttoPhos® AP Fluorescent Substrate System	S1000

Includes:

- 3 × 36mg AttoPhos® Substrate
- 4 × 60ml AttoPhos® Buffer
- 30ml AttoPhos® Calibration Solution

Product	Cat.#
AttoPhos® AP Fluorescent Substrate System Trial Size	S1001

Includes:

- 1 × 36mg AttoPhos® Substrate
- 1 × 60ml AttoPhos® Buffer

Storage Conditions: Store all components of the AttoPhos® AP Fluorescent Substrate System at 4°C protected from light.

AttoPhos® Substrate: AttoPhos® Substrate is supplied as a crystalline powder. Each vial contains 36mg (60µmol).

To reconstitute the AttoPhos® Substrate, carefully decant the entire contents of one 60ml bottle of AttoPhos® Buffer into the AttoPhos® Substrate to create a 1mM (pH 10) working solution. **Do not** attempt to transfer the solid into the buffer. **Do not** perform any further dilutions of the working solution. This solution is stable for at least one week when kept tightly covered in the dark at 4°C.

AttoPhos® Buffer: 2.4M DEA, 0.057mM MgCl₂, 0.005% NaN₃; (pH 10).

Four bottles, each containing 60ml of AttoPhos® Buffer, are provided. Three are used to reconstitute the AttoPhos® Substrate. The fourth bottle of Buffer can be used as a zero control (blank) or for further dilution of the AttoPhos® Calibration Solution.

AttoPhos® Calibration Solution: The AttoPhos® Calibration Solution (BBT) is supplied at a concentration of 500 ± 50ng/ml (or 1.75 × 10⁻⁶M) in 2.4M DEA (pH 10).

The Calibration Solution may be used to standardize instrumentation or quantitate the amount of BBT produced in a reaction. A broad range of concentrations may be prepared by diluting the Calibration Solution in AttoPhos® Buffer.

3. Assay and Standardization Procedure

1. For optimal results, the excitation wavelength should be in the range of 430–440nm. The emission wavelength should be greater than 525nm, with the range of 550–560nm optimal for systems requiring color discrimination. When values other than the optimal wavelengths are used, system sensitivity may decrease and/or background fluorescence increase.
2. Prepare a bottle of AttoPhos® Substrate by carefully pouring one entire bottle of buffer into the substrate bottle. **Do not attempt to transfer the solid into the buffer.** Replace the substrate bottle cap, and gently invert the bottle several times until the substrate is completely dissolved. Store the solution at 4°C, protected from light.

Note: Take the following precautions to reduce the potential for contaminating the AttoPhos® Substrate Solution with exogenous AP.

- **Do not** expose the solution to stir bars or any other mechanical stirring devices.
- **Do not** put pipette tips or any other dispensing device directly into the bottle of solution. Instead, pour the required quantity into a separate, clean container for pipetting into cuvettes or sample wells.

3. Assay and Standardization Procedure (continued)

3. Prepare unknowns and AP standards according to your established procedures.
4. To each unknown and AP standard, add a uniform amount of AttoPhos® Substrate Solution. This quantity should be determined experimentally and depends on the cuvette or multiwell plate used. Commonly 1–3ml for 1cm cuvettes or 100–200µl per well of a multiwell plate is required for ELISAs. All wells or cuvettes should contain identical total volumes.
5. Record the fluorometric values for each unknown and AP standard at the desired intervals (kinetics assay) or upon termination of the reaction (endpoint assay; 3,4).
6. Prepare a plot of the concentrations (or amounts) of AP standards versus fluorometric values.
7. Using the fluorometric value for each unknown, determine AP concentration by extrapolation on the standard curve generated in Step 6. If using an equation to describe the standard curve, apply the fluorometric values to the equation to achieve the AP concentration.

4. Example Titration of Calf Intestinal AP

The following example demonstrates detection of Calf Intestinal Alkaline Phosphatase (CIAP, Cat.# M1821). This protocol can be used to troubleshoot reagents and instrumentation applied to the fluorometric detection of AP with AttoPhos® Substrate.

1. A twofold dilution series of Calf Intestinal Alkaline Phosphatase in CIAP Dilution Buffer (Cat.# M1833) supplemented with 0.1% BSA was prepared over a range of 1×10^{-4} – 1.6×10^{-6} units/µl (at a specific activity of 2,350 units/mg). Control samples containing no enzyme also were prepared.
2. For each unit concentration, 10µl of CIAP was added to individual wells of a multiwell plate (Thermo Electron Microfluor®-2 black, flat bottom Microtiter® plate). Using a multichannel pipettor, 150µl of AttoPhos® Substrate was added simultaneously to each well.
3. The plate was incubated for 15 minutes at room temperature.
4. A CytoFluor® II fluorescent plate reader with a 450/50nm excitation filter and 580/50nm emission filter was used to scan the plate. The gain for this instrument was set at 55.
5. Fluorescence values for the no-enzyme control were subtracted from each enzyme amount to calculate the relative fluorescence units for each sample (Figure 3). The observed response is linear over the range of enzyme amounts examined. The limit of detection (determined as two standard deviations above the average control) was 2.9×10^{-7} units (or 0.9 attomoles).

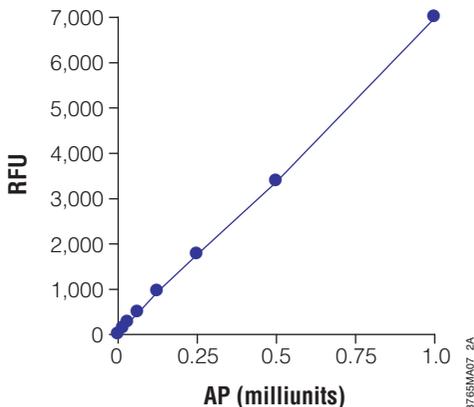


Figure 3. Fluorescence signal observed for a serial dilution of CIAP treated with 1mM AttoPhos® Substrate in a multiwell plate. The graph represents the increase in fluorescence per unit of AP at 15 minutes post-addition of AttoPhos® Substrate.

5. Interfering Substances

1. Ambient levels of AP are minimized by careful sample handling and acid treatment as described below. When analyzing very low levels of AP ($\leq 10^{-14}\text{M}$), any reagents used in an assay are suspect and must be checked for their effect (blank rate) on the system background drift. Treatment of containers and transfer devices with acid is recommended when the level of AP is near the lower limit of detection. Treatment with 0.1M HCl for at least 30 minutes followed by rinsing with freshly distilled water should sharply reduce or eliminate ambient levels of AP and contaminating metals that may contribute to high background levels.
2. The effect of trace quantities of metal (e.g., Fe) on the spontaneous hydrolysis of AttoPhos® Substrate can be a problem when testing for very low levels of AP ($\leq 10^{-14}\text{M}$). The source of metal ions should be maintained at the lowest possible practical level.

6. Instrumentation

Any fluorometer that can be set to an excitation wavelength of 430–440nm, and an emission wavelength greater than 525nm may be used. Suitable instrument sources include Bio-Tek, Thermo LabSystems and Molecular Devices.

7. References

The following references provide specific information on the application of AttoPhos® Substrate to a wide variety of assays that use fluorescence for AP detection.

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8. Related Products

Product	Size	Cat.#
AttoPhos® Substrate	36mg	S1011
	100mg	S1012
	1g	S1013
AttoPhos® Buffer	60ml	S1021
	240ml	S1022

©U.S. Pat. No. 5,424,440, Canadian Pat. Nos. 1,340,749 and 1,341,279, Japanese Pat. No. 3169216 and European Pat. No. 0 424 465.

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