

drug discovery

StarBright® Green Substrate: A Novel Fluorogenic Substrate for Alkaline Phosphatase Systems

By Pamela Hawkins, Jonathan Stephan,
Kyle Brueggeman, and Douglas Held
Sigma-Aldrich Corporation, St. Louis, MO, USA

Application Notes

- Superior sensitivity over most commonly used substrates
- Exceptional signal properties with no detectable photo-bleaching or photo-quenching
- Increased signal stability ≥ 1 year at 2-8 °C

Introduction

Alkaline phosphatase (AP) is commonly used as a detection enzyme for quantification or functional characterization of proteins or nucleic acids (mRNA). Optimal substrate properties for the detection of AP include increased sensitivity, signal stability and simple reaction kinetics for high-throughput applications. StarBright Green Substrate (Product Code [MT1000](#)) is a novel fluorogenic substrate for the accurate detection of AP activity. The substrate has an excitation wavelength of 440 nm and an emission wavelength of 505 nm, making it compatible with most common plate readers. The substrate is amenable to high-throughput formats and upon cleavage is converted to a reaction product that provides superior detection in the low attomole range (10^{-18} M or 2×10^{-7} units), displays a high quantum yield, offers flexibility for use in end-point or kinetic studies and is suitable for detection of various tagged fusion proteins including the commonly used FLAG® tag. Additionally, the substrate is also used in the High Performance Signal Amplification (HPSA™) mRNA Gene Expression Assays.

In this application note, we present a comparison of StarBright Green Substrate with other commercially available AP substrates. Detection limits and assay precision will be compared as well as the ability to detect a FLAG fusion protein. Data were collected on a Wallac VICTOR²™ plate reader (Perkin Elmer, Boston, MA). Fluorescence

results are in Relative Fluorescence Units (RFU) and the excitation and emission filters for each substrate are shown in Table 1.

Table 1. Excitation and Emission Filters for Substrates

Substrate	Excitation (nm)		Emission (nm)	
	Peak	Bandwidth	Peak	Bandwidth
StarBright Green	445	30	510	25
Substrate A	445	30	535	30
4-MUP	355	30	460	30

Superior sensitivity for AP detection

Detection sensitivity results from many factors including low K_m (7.5 μ M with calf alkaline phosphatase), high quantum yield, and high V_{max} (>100,000 per minute). Collectively, these factors lead to a reduction in spectral background while maintaining optimal signal generation. Figure 1 compares the initial velocity of AP substrates. Data calculations indicate that the enzyme-turnover rate of StarBright Green Substrate (107,000 molecules/minute) is 2.3 times greater than Substrate A and 23.5 times greater than 4-MUP.

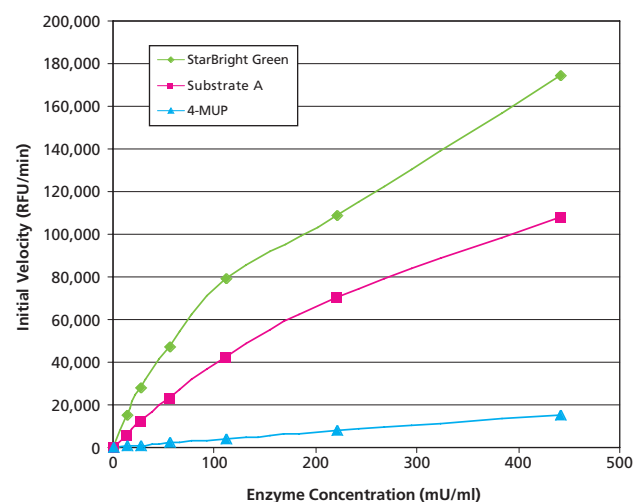


Figure 1. Initial Velocity Comparison of Alkaline Phosphatase Substrates. Calf alkaline phosphatase was diluted in Tris Buffered Saline and added at 10 μ l/well. Substrates were added at 100 μ l/well and plates were incubated at 37 °C. Fluorescent signals were monitored over 20 minutes and the initial rates of hydrolysis (RFU/min) measured. Results demonstrate the initial velocity for StarBright Green Substrate was 2.3 times faster than Substrate A and 23.5 times faster than 4-MUP.

The enzymatic constants were calculated for StarBright Green Substrate using enzyme at 1, 2 and 4 pg/well (1 pg = 0.28 mU/ml). Concentrations of StarBright Green Substrate varied from 4.5 to 36 μ M. Data were fitted to the Michaelis-Menton equation using BIOSOFT® EnzFitter Software (Ferguson, MO). Michaelis constants (K_m) and maximum velocities (V_{max}) were calculated. Data indicate

StarBright Green Substrate displays a low Michaelis constant and a high maximum velocity with calf alkaline phosphatase as shown in Table 2.

Table 2. K_m and V_{max} Values for Alkaline Phosphatase Substrates

Substrate	K_m (μM)	V_{max} (moles product/mole enzyme/minute)	Working Substrate Concentration (μM)
StarBright Green	7.5	107,000	10
Substrate A	300	>90,000	10,000
4-MUP	26.9	13,700	600

As shown in Figure 2, sensitivity can be enhanced by extending the incubation time of the substrate with the sample. Results indicate the StarBright Green Substrate generates greater signals than either Substrate A or 4-MUP. Detection limit (attomole/well) and assay precision of each substrate is presented in Table 3. Detection limit is the concentration of calf alkaline phosphatase that produces signals greater than background plus 2 times standard deviation. Lower concentrations of AP can be detected by extending incubation times. For example, at 90 minutes or longer, substantially lower quantities of AP can be detected with StarBright Green Substrate than with other substrates as shown in Table 4.

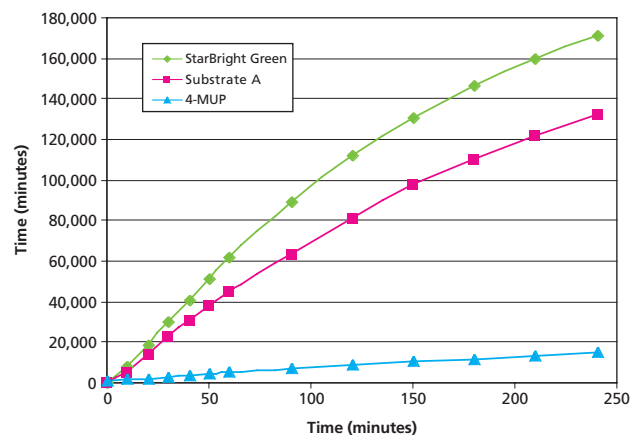


Figure 2. Time Course Comparison of Fluorogenic Substrates with Calf Alkaline Phosphatase at 1.7 mU/ml. Calf alkaline phosphatase was diluted in Tris Buffered Saline. Ten microliters was incubated with 100 μl of each substrate in a clear, multiwell plate at 37 $^{\circ}\text{C}$.

Table 3. Detection Limits and Precision Comparison of Substrates for Calf Alkaline Phosphatase

Substrate	Detection Limit	Precision
	Enzyme Quantity (attomole/well)	% CV at 1.4 attomole/well
StarBright Green	0.09	3.8
Substrate A	0.36	3.9
4-MUP	1.4	3.5

Table 4. Effect of Increasing Incubation Time on Detection Limit

Incubation Time (minutes) at 37 $^{\circ}\text{C}$	Detection Limit (attomole/well)		
	StarBright Green	Substrate A	4-MUP
30	1.42	1.42	11.4
90	0.09	0.36	1.42
120	0.09	0.36	1.42
180	0.09	0.18	1.42
240	0.04	0.18	0.71

Summary

Overall, data show that StarBright Green Substrate provides superior sensitivity over most commonly used substrates. Assays can be performed at 37 $^{\circ}\text{C}$ or room temperature and the signal is extremely stable (≥ 1 year at 2-8 $^{\circ}\text{C}$). StarBright Green Substrate is suitable for a variety of AP detection assays including the detection of mRNA in cell lysates and it has the greatest sensitivity, slope, and precision of all the substrates used in the HPSA β -Actin mRNA quantification assay. Additionally, it may be used for the detection of various tagged fusion proteins including the commonly used FLAG tag (Figure 3).

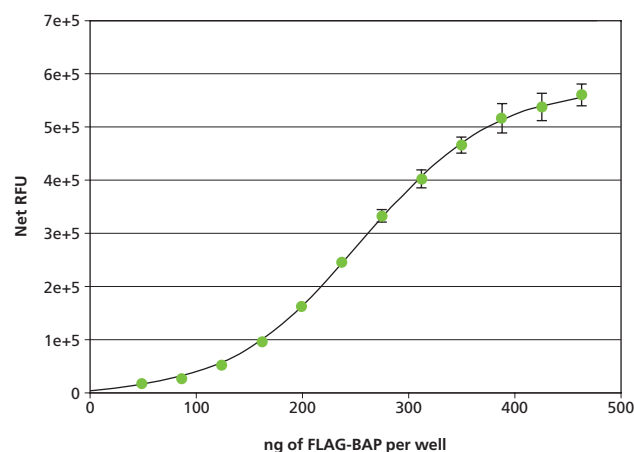


Figure 3. FLAG-BAP Detection Using StarBright Green Substrate. N-terminal FLAG-BAP control protein was diluted in Tris buffer with 1% Bovine Serum Albumin and incubated in a clear, 96-well Anti-FLAG M2 monoclonal antibody plate for 2 hours at room temperature. The plate was washed 4 times with TBS with 0.05% Tween-20 on a plate washer. StarBright Green substrate, pre-warmed to 37 $^{\circ}\text{C}$, was added and incubated at 37 $^{\circ}\text{C}$ for 10 minutes. The resulting fluorescence was read on a Wallac VICTOR² plate reader. Data is net RFU versus the 1% BSA blank.

Ordering Information

Product	Description	Unit
MT-1000	Alkaline Phosphatase Detection Kit (StarBright Green Substrate)	1 kit