Technical Bulletin 3

MicroPlate Detection comparison between SureLight® P-3L, other fluorophores and enzymatic detection

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I. Introduction

Achieving a very low limit of detection is a major goal in assay development. As the trend toward assay miniaturization continues in high throughput screening, the need for high sensitivity in the detection reagent is of increasing importance. Enzymatic amplification (in the form of alkaline phosphatase and horseradish peroxidase) systems have been used to challenge the radioactive gold standard. SureLight® PBXL dyes were developed to provide high sensitivity using prompt fluorescence as an alternative to other forms of amplification.(3) This study compared the sensitivity of one SureLight® dye, SureLight® P3L, to that achievable by methods employing enzymatic amplification as well as other fluorophores by prompt fluorescence. SureLight® P3L dye is SureLight® P3 that was modified by adding a polyethylene glycol linker arm between the proteinaceous dye and the specific binding agent covalently labeled with this dye. The SureLight® P3L has shown a large increase in sensitivity in flow cytometry when compared to other dyes and to the standard SureLight® P3.(2) Here, SureLight® P3L dye conjugate function was compared to a number of colorimetric and fluorogenic methods that use enzymatic processes to achieve an amplification of signal. SureLight® P3L dye compared well to all the methods tested, matching or beating the sensitivity of the other prompt fluorescent dyes and enzymatic methods in a biotinylated Mouse IgG/streptavidin conjugate model system in microplate format.

II. SureLight® P3L compared to other fluorescent dyes

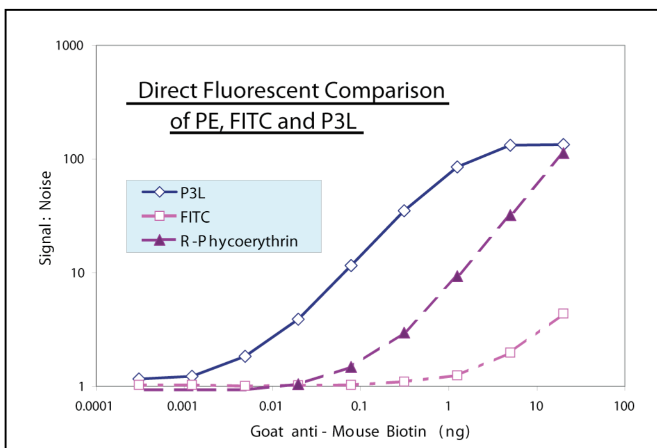

Figure 1 Comparison of the limit of detection of a Mouse IgG model system of SureLight® P3L, R-Phycoerythrin and FITC-labeled Goat anti-Mouse conjugates at 20 μg/mL as described in the Materials and Methods.

How sensitive SureLight® PBXL dyes are relative to other dyes used for prompt fluorescent detection is the most obvious question that needs to be answered when assessing a new dye. Fluorescein isothiocyanate (FITC) and R-phycoerythrin (R-PE) were chosen for this study for a variety of reasons. FITC was chosen since it is probably the most commonly used fluorescent dye, saving ethidium bromide, to which one can benchmark the intensity of a fluorescent dye. FITC is not a powerful dye, with an extinction coefficient around 10^4 M^1cm^{-1}, but is inexpensive and widely used. R-PE was chosen as an alternative prompt fluorescence standard because it is on the opposite end of the sensitivity scale to FITC, as the most intense fluorescent dye available prior to the SureLight® PBXL dyes, having an extinction coefficient in the 10^7 M^{-1} cm^{-1} range.(1) Direct or prompt fluorescence offers the advantage of instantaneous results and fewer steps to first result compared to enzymatic and radioisotopic detection methodologies. High sensitivity, prompt fluorescent dyes could provide the same sensitivity as radioactive and enzymatic methods with more rapid results and no expensive waste disposal.

Table 1 Comparison of the detection sensitivity of common fluorophores and enzymatic substrates in a biotinylated Goat anti-Mouse/streptavidin model detction system.

Detection Reagent	Biotin GAM IgG LOD (%CV)	Detection Method Measurement Parameters
BluePhos	1.4 pg/mL (7.6%)	colorimetric absorbance at 635 nm
ABTS	1.6 pg/mL (10.7%)	colorimetric absorbance at 410 or 650
Attophos	1.0 pg/mL (5.9%)	fluorogenic, fluorescence at 420ex/560em
R-PE	22 pg/mL (6.1%)	prompt fluorescence at 488ex/573em
FITC	400 pg/mL (3.7%)	prompt fluorescence at 485ex/515em
SureLight® P3L	1.3 pg/mL (9.9%)	prompt fluorescence at 590ex/666em

ex = excitation wavelength, em = emission wavelength

The comparison of the sensitivity achieved by SureLight® P3L, FITC and R-PE is shown in both Figure 1 and Table 1. It is clear from these data that the sensitivity levels of commonly used fluorochromes are easily bested by P3L conjugates. Statistical determination of the limit of detection for these three dyes showed the SureLight® P3L provides >16 times the sensitivity of R-PE and >300 times the sensitivity of FITC. It also seemed that there was additional room for optimization of the SureLight® P3L assay, as the %CV was almost 10 (Table 1).

III. SureLight® P3L sensitivity compared to enzymatic amplification methods

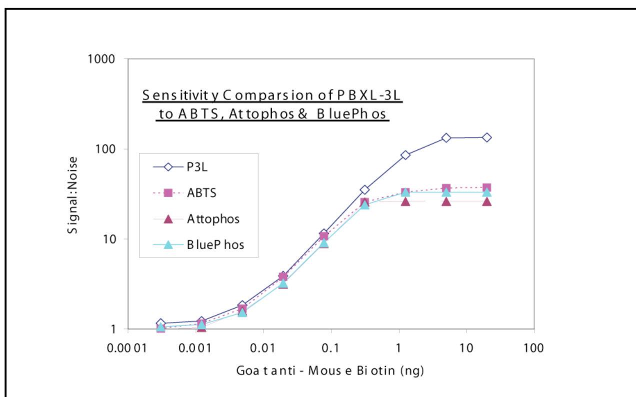

Figure 2 Sensitivity comparison of SureLight® P3L Goat anti-Mouse IgG conjugate prompt fluorescence detection to ABTS, Attophos and BluePhos enzymatic detection of Mouse IgG in a model system.

Conceptually, enzymatic amplification has an advantage over many other methods by the time factor added to any measurement: if you need more sensitivity, one can just incubate a while longer to obtain higher sensitivity. However, this comes with some tradeoffs in ease of use and time to first result. In addition, the enzymatic methods require the addition of enzyme, substrate and sometimes stop or developing solutions to maintain the product in a measurable form. A powerful fluorescent dye that provides sensitivity equal to the enzymatic methods would bypass the disadvantages of enzymatic methods without sacrifice of sensitivity.

Table 2 Concentrations of enzyme and fluorochrome in optimized detection format.

Amplification Method	**Detection Method (μg/mL)**					
	Attophos	**BluePhos**	**ABTS**	**FITC**	**R-PE**	**P3L**
Alk. Phosphatase	0.1	3	-	-	-	-
HRP	-	-	0.5	-	-	-
Prompt Fluorescence	-	-	-	20	20	20
Type	**Measuring Wavelengths (nm)**					
Excitation/Absorbance	425	635	405	485	550	590
Emission	560	n/a	n/a	525	590	660

Enzymatic assays were optimized for sensitivity, therefore depending on the given substrate different dilution factors of both enzymes (HRP and AP) had to be experimentally determined (Table 2). More sensitive substrates required much less enzyme than less sensitive substrates even though the overall detection limit did not vary much.

The data for the comparison of SureLight® P3L prompt fluorescent detection versus enzymatic detection methods is provided in Figures 2 & 3 and Table 1. From Table 1 the limit of detection can be easily compared between methods. The sensitivity achieved by the SureLight® P3L conjugate is in the same order of magnitude as the enzymatic methods. The Attophos™ method seems to be slightly more sensitive while the other methods seem to be slightly less sensitive than the SureLight® P3L method. The SureLight® P3L method is linear over three orders of magnitude while the enzymatic methods appear to be linear only over two orders of magnitude. On the truncated curve showing the lower end of the scale (Fig. 3), SureLight® P3L seems to give data that meet or exceed that of the enzymatic methods.

IV. Summary

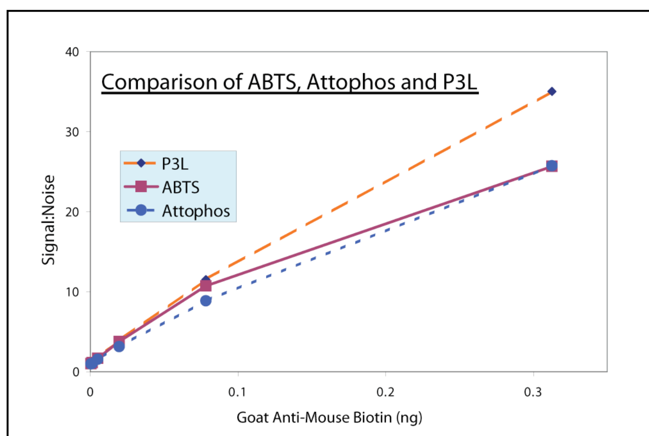

Figure 3 Comparison of SureLight® P3L prompt fluorescence detection to ABTS and Attophos enzymatic amplification on a truncated curve.

- ✓ SureLight® P3L conjugates provides sensitivity via prompt fluorescence that equals that achievable via a number of enzymatic methods (Attophos, BluePhos, and ABTS)
- ✓ SureLight® P3L conjugates easily beat the brightest competing direct detection fluor (R-PE) and commonly used FITC conjugates
- ✓ The SureLight® dyes offer greater ease of use and time to first result than enzymatic methods without sacrificing sensitivity achieved by enzymatic amplification.

V. Materials and Methods

The SureLight® P3L and R-PE streptavidin conjugates were from Columbia Biosciences. The BluePhos streptavidin detection kit was from KPL Laboratories. The Attophos detection kit was from Roche Diagnostics. The FITC conjugates were from Molecular Probes. The PBS used was 10 mM sodium phosphate (pH 7.4), 150 mM sodium chloride and 0.05% sodium azide. The Tween 20 was from Rolm & Haas. Bovine serum albumin was from Intergen. The 96 well Black Microplate (Dynex) was coated with 50 μg/ml of Mouse IgG (100 μL per well). The plate was coated at 4°C for at least 3 hours and overnight whenever possible. The plate was then washed with PBS, three times and patted out dry. The plate was blocked with 100 μL per well with standard blocking buffer (PBS plus 0.1% bovine serum albumin). Then, 1:4 serial dilutions for an initial 200 ng/ml dilution of biotinylated Goat anti Mouse-IgG were added at 100 μL per well. Samples were run in replicates of eight except that twenty wells were run without added biotinylated Goat anti Mouse IgG as negative controls to establish the limit of detection of each assay. The plate was then incubated for 1 hour at 37°C. The plate was then washed 3 times with PBS-Tween to remove unreacted biotinylated Goat anti Mouse IgG. SureLight® P3L was added at 20 μg/ml in 100 μL volumes to each well. The plate was then incubated for 1 hour at room temperature. The plate was washed and filled with 100 μL of PBS in each well and then read with excitation at 590 ± 45 nm and emission filter at 660 ± 32 nm. These data were analyzed (mean, sample standard deviation and coefficient of variance) and plotted in Excel. Trendlines were generated in Excel and linear equations were formulated using the plotted linear portion of the curve. Sensitivity (LOD) was defined as two standard deviations from the mean of the negative control. The y value at two standard deviations above the zero control was inserted into the equation $y = mx + b$ and the resulting x value was solved as the limit of detection of each assay. This LOD value determined the sensitivity of the assay at a 95% confidence interval ($P < 0.05$).

The prompt fluorescent detection comparisons were done at 20 μg/mL for all dyes tested using the coating and methodology described above for the enzymatic methods. Detection was done using a Fluorolite 1000 microplate reader (DYNEX) with the following filter sets: FITC (485 ± 22, 530 ± 30 nm), R-PE (550 ± 30, 590 ± 25) and SureLight® P3L (590 ± 45, 660 ± 32 nm).

The limit of detection (LOD) or assay sensitivity was generated using 20 replicates of the zero well and using two standard deviations from the mean to derive minimum detection limit.

VI. References Cited

1. **Rowan, K.** 1989. Photosynthetic pigments of algae. Cambridge University Press, Cambridge, UK.
2. **Telford, W., M. Moss, J. Morseman, and F. Allnutt.** 2001. Cyanobacterial stabilized phycobilisomes as fluorochromes for extracellular antigen detection by flow cytometry. J Immunol Meth.
3. **Zoha, S., S. Ramnarain, J. Morseman, M. Moss, F. Allnutt, Y.-H. Roger, and B. Harvey.** 1999. PBXL fluorescent dyes for ultrasensitive direct detection. Journal of Fluorescence **9**(3)**:**197-208.

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