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PBXL Fluorescent Dyes for Ultrasensitive Direct Detection

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PBXL™ dyes are a group of phycobilisome-based fluors that provide high sensitivity in direct fluorescent detection formats. Phycobilisomes are proteinaceous, supramolecular complexes that are photosynthetic antennae complexes in red algae and cyanobacteria. For the PBXL dyes, the phycobilisome has been chemically cross-linked in such a way that it remains water soluble and stable. Stabilized phycobilisomes (PBXL dyes) have high complex weights (between 10 and 15 million daltons) and Stokes shifts (up to 121 nm).They contain many hundreds of chromophores coordinated to transfer green and yellow light efficiently down an energy gradient to emit red light at wavelengths around 666 nm. Each PBXL dye can deliver up to 1400 chromophores per binding event without additional signal generation steps, signal amplification, or enzyme substrates. PBXL dyes provide a physical amplification of signal, enabling ultrasensitive, direct fluorescent detection of specific binding events. A number of studies done in our laboratory and in collaborating laboratories are summarized in this article. These studies demonstrated the utility of PBXL dyes in select high-sensitivity applications, such as a thyroid stimulating hormone (TSH) microplate immunoassay, detection of the low-density cell surface marker CD56 using flow cytometry, BSA–biotin, and actin detection in a western blot format and paternity testing using a DNA array on glass slides. PBXL dyes have the potential for providing a highly sensitive, simple, and direct fluorescent detection method to a wide range of targets and assay formats that could reduce costs associated with reagents and labor as well as decreasing the time to the first result.

KEY WORDS: Phycobilisomes; PBXL dyes; ultrasensitive detection; direct fluorescence.

INTRODUCTION

The PBXL™ dyes are a family of high molecular weight fluorescent pigments that deliver high sensitivity using direct fluorescent detection. Direct fluorescent detection offers speed and simplicity to any assay. However, direct fluorescence has been limited by the availability of dyes that deliver sufficient sensitivity. Complex procedures, such as enzyme amplification (e.g., chemiluminescence and polymerase chain reaction) and the use of radioisotopes, have been required to obtain clinically relevant sensitivity. PBXL dyes can eliminate amplification steps while maintaining the desired sensitivity levels. PBXL dyes are derived from phycobilisomes, the light-harvesting photosynthetic antennae complexes of red algae and cyanobacteria [1–4]. The phycobilisome is composed of phycobiliproteins and colorless linker polypeptides arranged optimally for the transfer of green and yellow light energy down a gradient with emission as red light. Phycobiliproteins, which are components of the PBXL dyes, are used extensively in sensitive direct fluorescent detection [5–7]. The PBXL dyes build on the intensity of the phycobiliproteins by having many of them

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within the complex to extend the utility of direct fluorescence to assays, which now require enzyme-linked or radioisotopic procedures. For the PBXL dyes, the entire phycobilisome has been chemically cross-linked in such a way that it remains water soluble and stable[8]. These stabilized phycobilisomes (PBXL dyes) have large complex weights (between 10 and 15 million daltons) and Stokes shifts (up to 121 nm).

A physical amplification of signal is achieved with the PBXL dyes. In contrast to the phycobiliprotein R-phycoerythrin (R-PE), which delivers 34 chromophores per binding event [4], each PBXL supramolecular complex can deliver up to 1400 chromophores per binding event. This large number of chromophores provides increased signal, permitting sensitivity not possible with current direct detection technologies. Moreover, the enhanced PBXL fluorescent signal is accomplished without signal triggering or generating steps, enzyme and substrates or additional signal amplification.

PBXL dyes can be used in a variety of fluorescent assay formats to provide high-sensitivity, direct fluorescent detection. Assays that have already been evaluated include high sensitivity microplate immunoassays, flow cytometry, western blots, and DNA arrays. The PBXL dyes have the combined advantages of ease of use, low cost, stability, and high sensitivity that establish a new standard of performance for direct fluorescent detection. PBXL technology could enable lower cost instrumentation and reagents, improved sensitivity, and a greater degree of microarray miniaturization when using direct fluorescent detection.

PHYSICAL AND SPECTRAL CHARACTERISTCS

The PBXL dyes are unique fluors that are proteinaceous, have high molecular weights, and contain many chromophores protected within their protein structure. The unique properties of these fluors are reviewed here to orient better those interested in using these pigments. The two dyes currently available, PBXL-1 and PBXL-3, are produced from red and blue–green algae, respectively. The isolation and stabilization procedure involves breaking of the cells, differential polyethylene glycol precipitation, size exclusion column chromatography, and chemical cross-linking [8]. Stabilized PBXL dyes are stored at 4°C in 0.75 *M* potassium phosphate (pH 7.2) with 0.05% sodium azide until used for conjugate production. The pigments and their conjugates are stable for years when stored at 4°C in the dark.

The spectral properties of the PBXL dyes distinguish them from other dyes. They have large Stokes shifts and emit in the red. PBXL-1, the stabilized *Porphyridium cruentum* phycobilisome, has an excitation spectrum that reflects its constituent phycobiliproteins (Fig. 1A), which are B-phycoerythrin (B-PE), R-phycocyanin (R-PC), and allophycocyanin (APC). PBXL-3, the stabilized *Spirulina platensis* phycobilisome, has an excitation spectrum reflecting the C-PC and APC of the native phycobilisome (Fig. 1B). The fairly broad excitation peaks allow the use of a number of commercially available lasers, such as the argon and helium–neon (HeNe) lasers. The emission profiles also reflect a coupling of energy transfer from PE to PC to APC for emission from APC or a specialized APC.

The large Stokes shifts of these dyes are a distinct advantage for the signal-to-noise ratios that can be achieved. The Stokes shift of PBXL-1 is 121 nm. However, when PBXL-1 is excited at 488 nm (the argon laser line), the shift is 178 nm with about 36% of the maximum intensity (Table I). There is a predictable leak at the B-PE fluorescence emission maximum (573 nm) that is roughly one-fourth the intensity of that at 666 nm. This

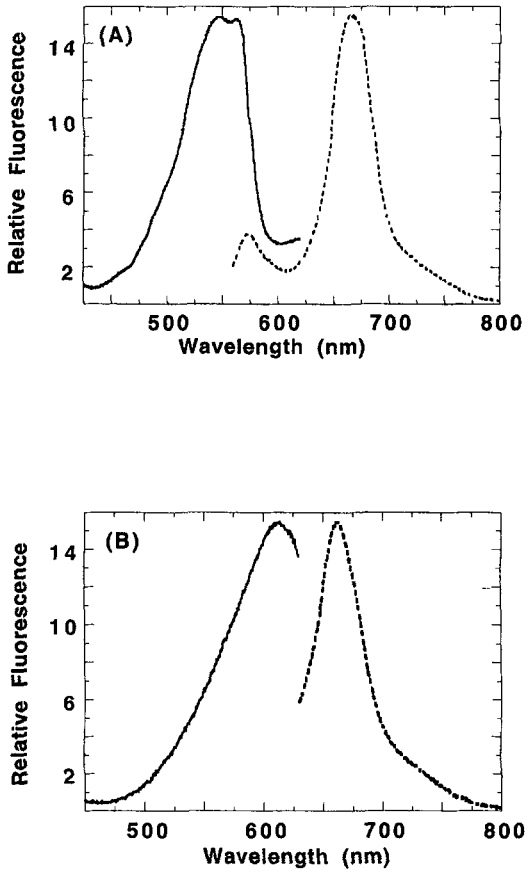

Fig. 1. Fluorescence emission and excitation spectra for PBXL-1 (A) and PBXL-3 (B). Excitation spectra (——) and emission spectra (——). Spectra are normalized and uncorrected.

Table I. Selected Physical and Spectral Properties of PBXL-1 and PBXL-3

Dye	Excit. max (nm)	Emiss. max (nm)	MW (Da)	% fluorescence relative to excitation maximum at important laser lines[a]				
				488 nm	543 nm	594 nm	612 nm	633 nm
PBXL-1	545.0	666.0	$\approx 1.5 \times 10^7$	36.3	97.1	17.5	16.3	15.5
PBXL-3	614.0	622.0	$\approx 1.0 \times 10^7$	6.8	37.3	88.5	93.2	65.8

[a] Argon laser line at 488 nm. The 543-, 594-, 612-, and 633-nm laser lines are for the HeNe laser.

leak appears to be predictable and compensation should be possible. Little or no leak of C-PC emission is apparent in PBXL-3 (Fig. 1B), which provides a 48-nm Stokes shift.

Equations derived by MacColl and Guard-Frair for common mixtures of phycobiliproteins [4] were used to determine the probable number of B-PE, R-PC, and APC molecules in each PBXL-1 complex. Using these equations, the *P. cruentum* derived PBXL-1 was estimated to have (36) B-PE, (6) R-PC, and (6) APC molecules. The number of chromophores in each of these phycobiliproteins has been published [4] and indicate that PBXL-1 will contain 1400 chromophores.

To be useful in direct detection, PBXL dyes needed to be soluble and stable under conditions normally used in immunoassays. This is not possible with native phycobilisomes, which, while soluble, dissociate at low salt concentrations. Chemical stabilization prevented dissociation but had to be carefully controlled to maintain solubility. Since PBXL dyes are supramolecular complexes, they can be spun down by centrifugation. The amount of PBXL dye remaining in solution after low-speed centrifugation was used to characterize a "functional solubility," meaning that they remain in solution under normal assay conditions. PBXL-1 remained almost totally in solution (>90%) after centrifugation at 5000*g* for 5 min. Significant losses (up to 40%) of PBXL-1 were observed after centrifugation for 5 min at 12,000*g* and greater (Fig. 2).

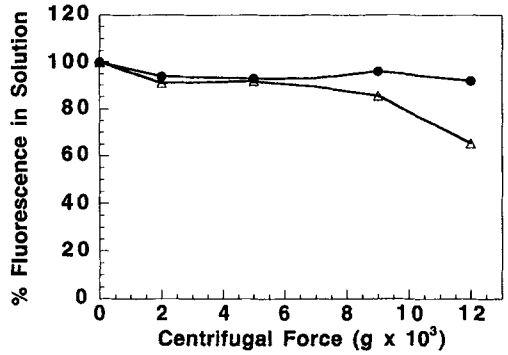

Fig. 2. Percentage of PBXL-1 remaining in solution after a 5-min spin at various centrifugal forces. PBXL-1 (△——△); PBXL-3 (●——●).

PBXL-3 had similar solubility. The PBXL dyes, while capable of being spun down, remain in solution (i.e., functionally soluble) under conditions commonly used for immunoassays. Streptavidin and antibody conjugates retain similar solubility characteristics making them suitable for specific binding assays.

The complex structure of the phycobilisome supramolecular complex has been previously reported [2]. Electron microscopy was used to determine if chemical cross-linking modified the structure of the PBXL dyes compared to previously reported phycobilisome structures. Transmission electron microscopy (TEM) was done by Structure Probe (West Chester, PA) using negative staining techniques previously applied to phycobilisomes [2]. PBXL-1 appears as regular hemispherical-shaped structures with an approximate diameter of 50 nm [2]. This agrees well with the previous literature for native phycobilisomes from *P. cruentum*. PBXL-3 has a linear structure that, on its largest diagonal, is about 80 nm long. This agrees with the expected shape of the native phycobilisomes of this cyanobacteria, which is hemidiscoidal [3].

The PBXL dyes have been conjugated to a variety of binding partners that include streptavidin, biotin, antibodies, nucleic acids and peptides. Glutaraldehyde, SPDP, NHS-ester, and sulfo-SMCC/SATA conjugation procedures have all been successfully employed. With the exception of the Amersham–Pharmacia Biotech PBXL-3 conjugates, the applications described in this paper employed conjugates made by a heterobifunctional procedure whereby the sulfo-SMCC-modified binding partner was added to the SATA-modified PBXL dye. The conjugation procedure used for the Amersham–Pharmacia conjugates is considered proprietary and is not discussed. The ratios of binding partner to PBXL dye were empirically determined to give optimal assay performance.

APPLICATION TO CLINICAL IMMUNODIAGNOSTICS

A detection system that bypassed a number of amplification steps but still provided clinically relevant sensi-

tivity would have a significant impact on clinical immunodiagnostics. It would lower the cost of assay reagents as well as lowering the cost of instrumentation (i.e., less fluid handling, robotics and software). To demonstrate the utility of PBXL dyes in a clinical setting, thyroid stimulating hormone (TSH) was chosen as a clinically important analyte that requires a high sensitivity for detection. For detection technologies to be useful for automated clinical immunochemistry systems, they must first demonstrate the ability to perform a clinically relevant high-sensitivity assay such as TSH assay (0.05 μIU/ml). The PBXL technology demonstrated a high level of sensitivity (6.2×10^{-14} *M* or 0.01 μIU/ml). This level of sensitivity had been possible until now only with detection technologies that involve enzymatic signal processing or signal triggering on automated instrumentation (e.g., chemiluminescence or chemifluorescence) or radioisotopic methods. This study shows that a simple direct fluorescent approach is possible with PBXL dyes and an abstract of this work has been presented [9].

To do this PBXL-based fluorescent immunoassay for TSH, the wells of a black 96-well plate (Dynex Technologies, Chantilly, VA) were first coated with an anti-TSH-β capture monoclonal (Fitzgerald Industries International, Concord, MA). The antibody was diluted to 100 μg/ml in plate coating buffer [100 m*M* sodium phosphate (pH 7.4), 150 m*M* sodium chloride, 0.05% sodium azide]. One hundred fifty microliters of the coating solution was added to each well. The plates were covered and incubated overnight at room temperature. The coating solution was aspirated and the plates were washed three times with 350 μl/well wash buffer [100 m*M* sodium phosphate (pH 7.4), 150 m*M* sodium chloride, 0.05% sodium azide, 0.05% Tween 20]. The plates were then blocked with 350 μl/well blocking buffer [100 m*M* sodium phosphate (pH 7.4), 150 m*M* sodium chloride, 0.05% sodium azide, 0.05% Tween 20, 1.0% bovine serum albumin] for 2 h at 37°C. The wells were aspirated then washed four times with 350 μl/well wash buffer and used immediately. Calibrators (TSH standards) were made by adding purified TSH (Scripps Laboratories, San Diego, CA) to a calibrator matrix [100 m*M* sodium phosphate (pH 7.4), 150 m*M* NaCl, 6.0% bovine serum albumin]. The anti-TSH-β monoclonal antibody (AbProbe International, Portland, ME) was conjugated to PBXL-1 through SATA/sulfo-SMCC heterobifunctional cross-linking chemistry (Pierce, Rockford, IL) at an offered molar ratio of 18:1, antibody to PBXL-1, respectively.

In this sandwich monoclonal immunoassay, 75 μl TSH calibrator and 75 μl assay buffer [100 m*M* sodium phosphate (pH 8.4), 150 m*M* sodium chloride, 0.05% sodium azide, 0.05% Tween 20, 1.0% bovine serum albumin] were added to each well. The plates were covered and incubated for 90 min at 37°C, then washed three times with 350 μl/well wash buffer. The anti-TSH:PBXL-1 conjugate was diluted to 100 μg/ml (as pigment) in reagent dilution buffer [100 m*M* sodium phosphate (pH 8.4), 150 m*M* sodium chloride, 0.05% sodium azide, 1.0% bovine serum albumin]. Then 100 μl assay buffer and 50 μl anti-TSH:PBXL-1 conjugate (100 μg/ml in reagent dilution buffer) were added to each well. The plates were covered and incubated for 90 min at 37°C, then washed three times with 350 μl/well wash buffer. Plate coating buffer (100 μl) was added to each well. The fluorescence was measured on a Fluorolite 1000 plate reader (Dynex Technologies, Chantilly, VA) at 10 V using 550-nm (±15-nm-bandpass) excitation and 660-nm (±16-nm-bandpass) emission filters.

The PBXL-based TSH immunoassay was characterized for standard curve linearity, assay precision and analytical detection limit. The five-point calibration curve (0.0, 0.05, 1.0, 5.0, and 10.0 μgIU/ml) employed a weighted logit fit analysis (Fig. 3A). A truncated standard curve is also provided at low analyte levels, between 0.0 and 0.2 μIU/ml (Fig. 3B). Linearity and the limit of quantitation were determined by assaying dilutions of the 10 μIU/ml TSH calibrator in the zero calibrator matrix (10.0, 5.0, 2.5, 1.0, 0.5, 0.2, 0.1, 0.05, 0.01, 0.005, and 0.0 μIU/ml) in replicates of six. Concentrations were determined from a five-point calibration curve. Linear regression analysis of all points demonstrated a slope of 0.983 (1.010 to 0.956, upper and lower 95% confidence interval), a *y*-intercept of 0.019 μIU/ml (0.110 to −0.071 μIU/ml, upper and lower 95% confidence interval), standard error of the estimate of 0.334 μIU/ml and an r^2 of 0.996. Quadratic regression analysis for curvature demonstrated a *P* value of 0.06, indicating lack of evidence for significance of line curvature. The limit of quantitation was 0.01 μIU/ml. This was the lowest dilution that demonstrated a statistically significant difference ($P \leq 0.05$) from the zero calibrator as determined by a nonpaired Student's *t*-test analysis. The analytical limit of detection of 0.01 μIU/ml was calculated from two times the standard deviation of 20 replicates of the 0 μIU/ml calibrator. This translates to 1.5 pg/ml or 6.2×10^{-14} *M* sensitivity. The within-run imprecision (CV) for replicates of 20 was 6.4 and 4.5% CV at concentrations of 0.56 and 7.2 μIU/ml, respectively. The dynamic linear assay range was 0.01 to 10.0 μIU/ml.

Optimization of this assay toward more rapid incubation times has not yet been investigated. Hypothetical comparison to other systems indicated that shorter incubation times should not provide an obstacle to obtaining high sensitivity.

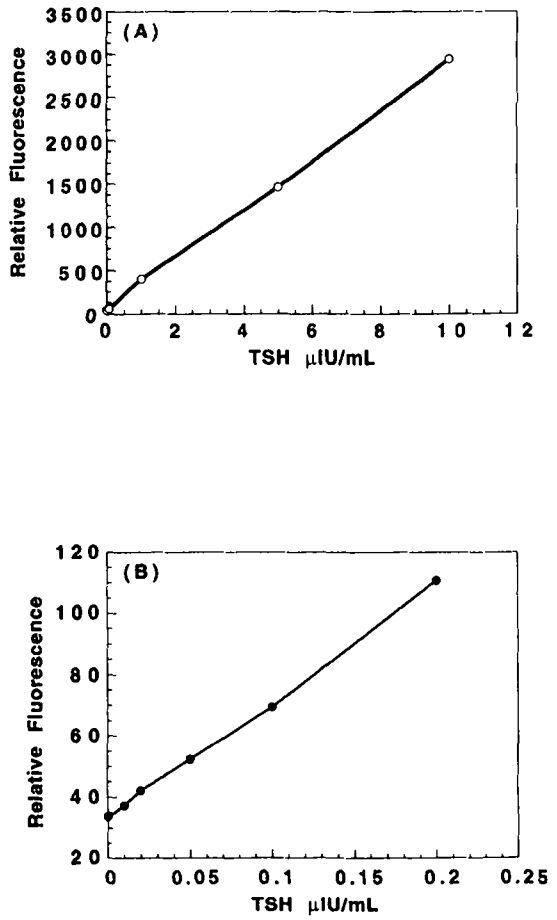

Fig. 3. PBXL-1-based direct fluorescent immunoassay for TSH using anti-TSH monoclonals in a sandwich format. (A) A five-point calibration curve between 0.0 and 10.0 µIU/ml. (B) A truncated curve focused on 0.0 to 0.2 µIU/ml, providing additional data points.

Femtomolar (6.2×10^{-14} *M*) detection in a sandwich monoclonal-based immunoassay has been possible only with indirect detection technologies that involve signal activation or enzymatic signal generation (e.g., chemiluminescence or chemifluorescence). PBXL dyes enabled femtomolar detection even within the limitations of a manual assay. Both the detection limit and the assay precision might substantially improve if the assay were formatted on automated instrumentation specifically optimized for the features of the PBXL dyes.

APPLICATION TO WESTERN BLOTTING

A simplified detection system that eliminated amplification steps but still provided adequate sensitivity would have a positive impact on the advancement of blot detection methodologies. It would lower the cost of assay reagents, decrease the cost of labor, and shorten the time required to get the data. Direct fluorescent blot detection methods have been neglected due to a lack of sensitivity and high background. The availability of scanning imaging instrumentation (e.g., FMBIO II and STORM 860) makes use of fluorescent dyes a real possibility. Summarized here are data from our lab with the FMBIO II system and data from Amersham–Pharmacia Biotech using their STORM 860 system and PBXL-3 based dyes.

PBXL-1 on the FMBIO II System. PBXL-1 titration and BSA–biotin western blots were used to demonstrate the ease of use and sensitivity of the PBXL-1 dye in western blotting. This dye works optimally with the FMBIO II (Hitachi Software Engineering Co., South San Francisco, CA) laser, which excites at 532 nm and allows quantitative direct fluorescent detection at low levels (attomolar).

In an effort to estimate the potential PBXL-1 sensitivity without binding chemistry, a dilution series of PBXL-1 was immobilized on Hybond-ECL, a nitrocellulose membrane (Amersham Pharmacia Biotech, Inc., Piscataway, NJ), to determine the optimal molar sensitivity and linear dynamic range. Hybond and ECL are trademarks of Amersham Pharmacia Biotech Limited or its subsidiaries. PBXL-1 was diluted in 100 m*M* sodium phosphate (pH 7.4), 150 m*M* NaCl, 0.1% BSA (PBS100B), then 0.5-µl aliquots were manually applied to nitrocellulose. Samples were dried for 10 min, immersed in 100 m*M* sodium phosphate (pH 7.4), 150 m*M* NaCl (PBS100), then imaged on the FMBIO II using a 650-nm (15-nm-bandpass) filter.

The lower limit of visible PBXL-1 detection was 0.78 pg, equal to 5.20×10^{-20} mol or 31,300 molecules. The dynamic linear range was approximately three orders of magnitude, between 1 ng and 1.6 pg (Fig. 4). The linear portion of the PBXL-1 dilution curve had an r^2 of

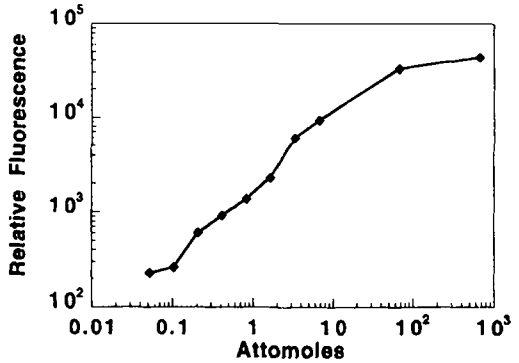

Fig. 4. PBXL-1 titration on Hybond-ECL (nitrocellulose membrane) detected directly with the FMBIO II imaging system.

0.980. This sensitivity was not optimal, since the 650-nm-bandpass filter captured only 30% of the PBXL-1 emission. A 630-nm bandpass filter increased the lower limit of detection to 0.195 pg (1.3×10^{-20} mol or 7830 molecules) but had an adverse effect on the linear dynamic range and was not used extensively. The area imaged for each spot was calculated from the estimated 0.75-mm spot radius. The lower limit of detection per unit area was calculated to be 0.0044 PBXL-1 molecule per μm^2 or 1 PBXL-1 molecule per 226 μm^2.

To test the sensitivity of PBXL-1 conjugates in western blot detection using the biotin/avidin system, BSA–biotin was diluted in PBS100B and 0.5-μl aliquots were manually applied to Hybond-ECL. The amount per blot was from 100 ng to 0.1 pg. Samples were air-dried for 5 min and then blocked in 1.5% BSA, 1% casein, 0.5% gelatin, 0.1% Tween 20 in PBB100 (MBB buffer) for 1 h at room temperature. Streptavidin (Prozyme, Palo Alto, CA) was conjugated to PBXL-1 through SATA/sulfo-SMCC heterobifunctional cross-linking chemistry (Pierce) at an offered ratio of 12:1 streptavidin to PBXL-1, respectively. The streptavidin:PBXL-1 conjugate was diluted to 10 μg/ml in MBB and incubated with the membranes for 30 min at room temperature with shaking. Membranes were washed with PBS100 with 0.1% Tween 20 (PBST) and scanned on the FMBIO II.

The lowest amount of BSA–biotin observed was 0.78 pg or 1.11×10^{-17} mol (Fig. 5). The linear dynamic range of detection was approximately two orders of magnitude (0.78 to 50 pg) with an r^2 value of 0.9715. Additional sensitivity could be obtained through further optimization. The three orders of magnitude difference between the theoretical limit of detection of PBXL-1 and experimental results for the biotin/avidin system indicates that some improvement may be possible.

PBXL-3 on the STORM 860 System. An actin dot-blot titration and actin western blots were used to demonstrate the ease of use and sensitivity features of the PBXL-3 dye. This dye works optimally with the STORM 860 System (Molecular Dynamics, Inc., Sunnyvale, CA) laser, which excites at 635 nm and makes direct fluorescent detection quantitative at low concentrations.

To titrate the level of PBXL-3 sensitivity with antigen–antibody binding chemistry, a dilution series of actin from chicken gizzard (Sigma, St. Louis, MI) was immobilized on Hybond-C pure membranes (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) to determine the linear dynamic range. Rabbit antiactin (Sigma, St. Louis, MO) was diluted 1:100 in 10 m*M* sodium phosphate (pH 7.5), 0.1% Tween 20 (PBS Tween 20). PBXL-3-labeled goat anti-rabbit IgG (Amersham Pharmacia Biotech, Inc.) was diluted 1:500 in PBS Tween 20. Membranes were processed according to the package insert for PBXL-3 labeled goat anti-rabbit IgG, then excited and imaged with the 635-nm laser on the STORM 860 System. A dynamic linear range between 0.5 and 250 ng was demonstrated (Fig. 6) with the actin blot blot. It should be noted that phosphate levels below 50 m*M* are not optimal for these dyes and modified PBS that contains 100 m*M* phosphate is recommended. Slight improvements in the data are therefore possible with the current buffers.

The actin model system was used to compare the sensitivity obtained from PBXL-3 and enzymatic chemifluorescence (Vistra ECF) detection systems in a western blot format. Vistra ECF reagents include an alkaline phosphatase (AP)-labeled goat anti-rabbit, Vistra ECF substrate, and Vistra substrate dilution buffer (Amersham Pharmacia Biotech, Inc.). Vistra ECF is a trademark of Amersham Pharmacia Biotech Limited or its subsidiaries. For this comparison, Rainbow molecular weight markers (Amersham Pharmacia Biotech, Inc.) in lane 1, 10 ng of actin in lane 2, and serial dilutions (1:50, 1:100, 1:200, and 1:400 in PBS Tween 20) of a rat brain homogenate in lanes 3 through 6 were electrophoresed on a resolving gel for 1 h at 100 V. The gels were electroblotted onto Hybond-C pure membranes according to the package insert. Rabbit antiactin (Sigma), Vistra ECF reagents, and PBXL-3-labeled goat anti-rabbit (Amersham Pharmacia Biotech, Inc.) were employed for the detection of actin according to the respective package inserts. Membranes were scanned on the STORM 860 scanner. A lower limit of detection was not calculated since actin concentrations were not determined. Nonetheless, the PBXL-3 conjugate was able to detect the same lowest detectable dilution (1:400) of the rat brain homogenate as the ECF detection system (Fig. 7). This is the most sensitive direct fluorescent detection method available. PBXL-3 conjugates to goat anti-mouse, goat anti-rabbit, and streptavidin are commercially available through Amersham Pharmacia Biotech Inc. for use in western blots.

Western blots have typically avoided direct nonradiometric detection methods due to a lack of sensitivity and high background. PBXL-1 was imaged by the FMBIO II at a lower limit of detection, 1 PBXL-1 molecule per 226 μm^2. PBXL-1 dye provided sensitive, direct fluorescent detection of proteins immobilized on membranes with one fewer reagent, two fewer steps, and a time to the first result shorter by 3 to 24 h relative to colorimetric detection. PBXL-3 enabled sensitive detection of proteins immobilized on membranes with two fewer reagents and two fewer steps with a comparable sensitivity and time to the first result relative to enzymatic chemifluorescence detection. The PBXL technology creates the potential for direct multicolor fluorescent blot

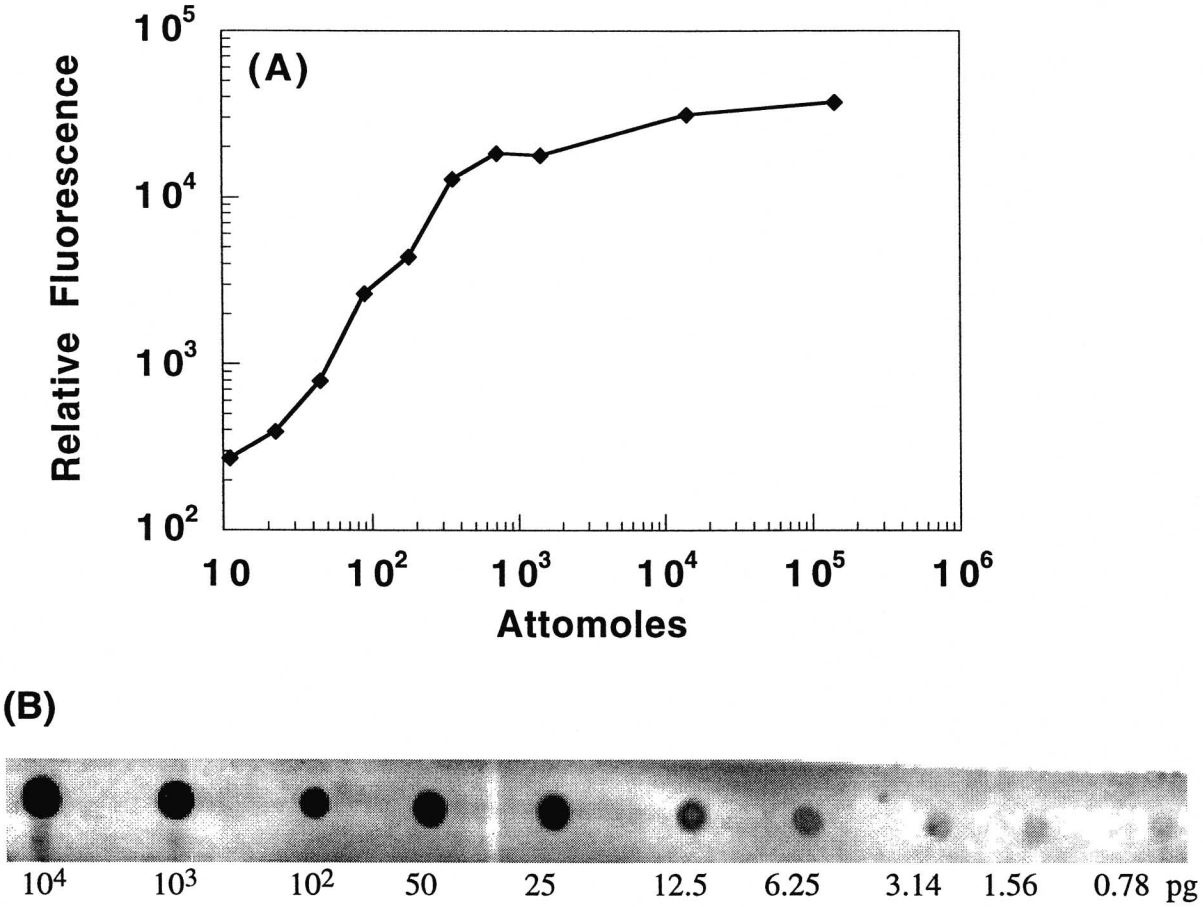

Fig. 5. BSA–biotin detection on nitrocellulose with PBXL-1:streptavidin. (A) Graphical representation of BSA–biotin titration curve; (B) FMBIO image of the data.

detection and is the most sensitive direct fluorescent blot detection method available.

APPLICATIONS TO DNA ARRAYS

Direct detection and high sensitivity are the goal for DNA arrays. Multiple steps and other forms of amplification are often used as a last resort to achieve the desired sensitivity. As these arrays become more miniaturized, direct detection will increase in importance as both detection limits are reached and the ability to do indirect assays is compromised by very small volumes.

PBXL-1 dye has been applied to DNA arrays in a paternity testing application. Direct fluorescent detection with PBXL-1 enables sensitive detection of DNA immobilized on glass slides. The following data demonstrate the detection of single nucleotide polymorphisms using a solid phase primer extension method by Genetic Bit Analysis (GBA) [10, 11]. These data were generated on the FMBIO II by Molecular Tool Inc. (MTI). Martek and MTI continue to optimize this system for paternity testing as well as other DNA array applications.

For the DNA array analysis, each slide had four 11-mm Teflon outlined squares. GBA primers were used for a 10-human marker panel and were patterned in a 4 × 4 array setup by a Hamilton 2200 robot using proprietary attachment chemistry. The array configuration numbers in Fig. 8A represent the locus numbers of the primers. The droplet size was 50 nl and the spacing between droplets was 2 mm. A fluorescein-labeled oligo served as an attachment control (M). One slide of four squares consisted of the PCR multiplex mix for the father (Coriel DNA 1), one for the mother (Coriel DNA 2), one for the daughter (Coriel DNA 3), and an unrelated control of placental DNA. Single-stranded PCR products generated by T7 exonuclease treatment were applied to the arrays [10]. The primers immobilized on the arrays were

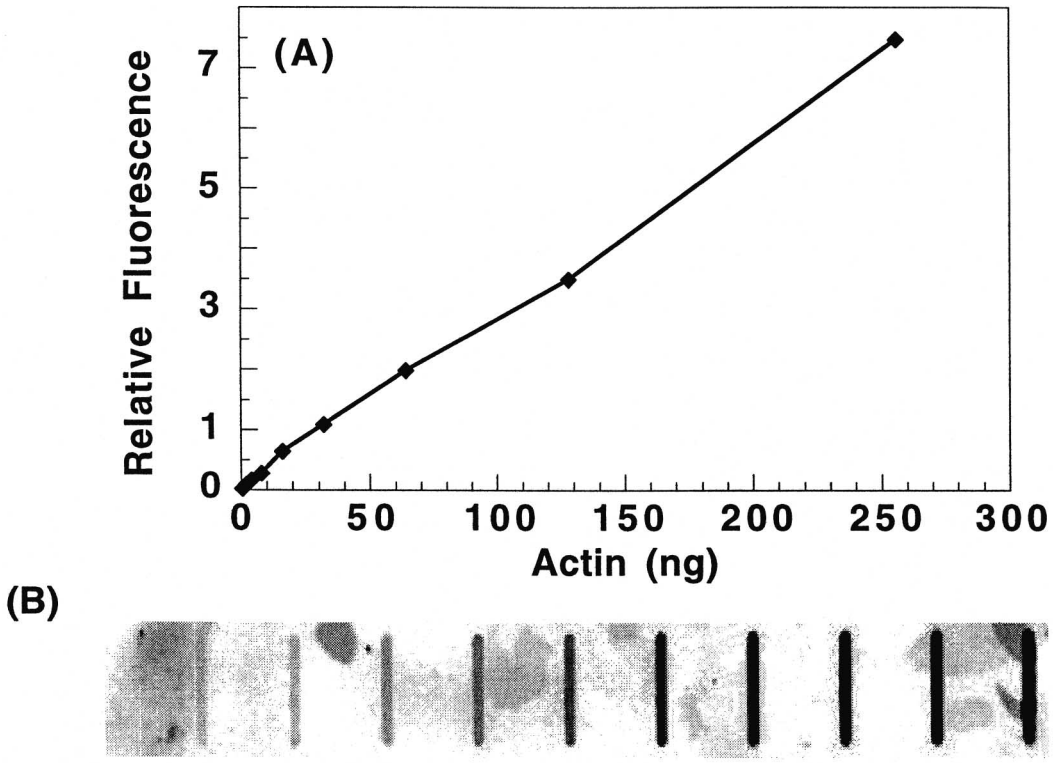

Fig. 6. Actin detection on Hybond-C pure membranes with PBXL-3 labeled goat anti-rabbit and rabbit antiactin. (A) Graphical representation of actin titration; (B) STORM system image of the data.

designed to hybridize to the single-stranded target DNA immediately adjacent to the polymorphic site of interest. The slides were incubated for 1 h at room temperature for hybridization. The slides were washed with 10 m*M* Tris–HCl (pH 7.5), 150 m*M* NaCl, and 0.05% Tween 20

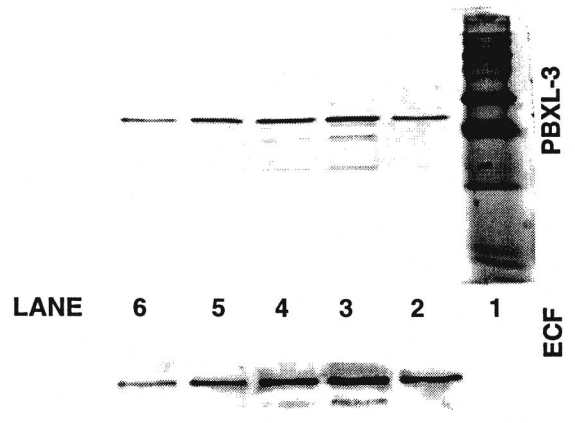

Fig. 7. Actin detection on Hybond-C pure membranes comparing PBXL-3 and ECF detection systems.

(TNTw), then the specific extension mix for each of the four bases was added to the appropriate squares [11]. Each extension mix consisted of one biotin-labeled ddNTP, three unlabeled ddNTPs, GBA extension reaction buffer, Mn^{2+}, and exonuclease-free Klenow polymerase. The primer is thereby extended at the 3′ end by one biotinylated ddNTP in the presence of all four chain-terminating ddNTPs. The slides were incubated for 30 min at room temperature, then washed with 0.1 *N* NaOH and TNTw. Streptavidin (Prozyme) was conjugated to PBXL-1 through SATA/sulfo-SMCC heterobifunctional cross-linking chemistry (Pierce) at an offered molar ratio of 12:1, streptavidin to PBXL-1, respectively. The streptavidin:PBXL-1 conjugate was diluted to 100 μg/ml in phosphate-buffered saline (pH 7.4) plus 0.1% BSA. The slides were treated with 200 μl per slide of streptavidin:PBXL-1 conjugate for 1 h. The PBXL-1:streptavidin conjugate bound to the incorporated biotinylated bases. The arrays were washed with TNTw three times, then with distilled deionized water one time before imaging on the FMBIO II. The PBXL signal was imaged at 650-nm emission. The fluorescein was imaged at 505-nm emission (data not shown).

(A)

M	12	20	M
21	M	27	45
60	84	M	94
M	210	212	M

(B)

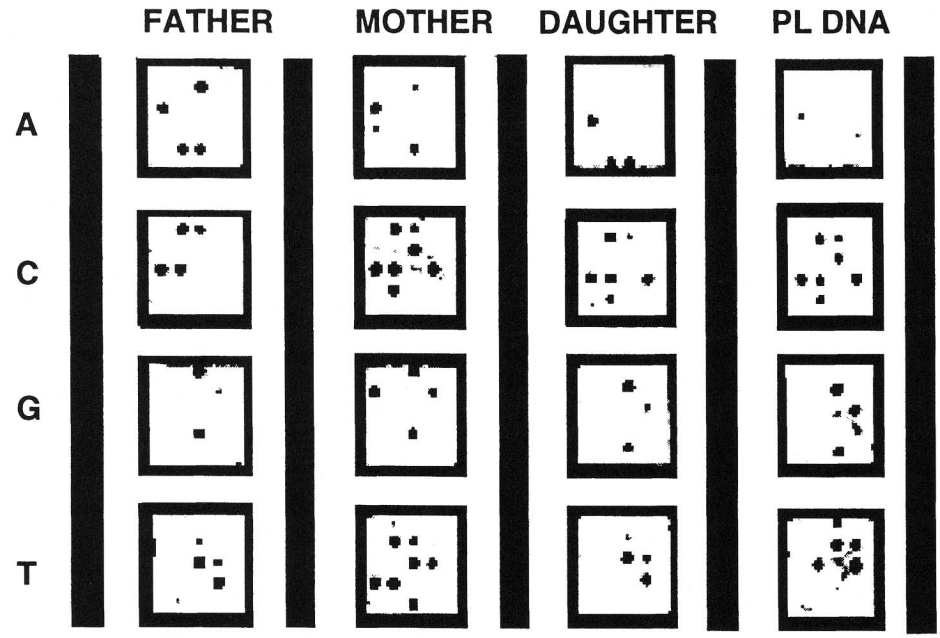

Fig. 8. Genetic bit analysis for single-nucleotide polymorphisms using PBXL-1-based direct detection and the FMBIO imaging system. (A) Pattern of 4 × 4 microarray for GBA primers. (B) Father, mother, daughter, and placental DNA control GBA arrays detected with streptavidin:PBXL-1 and imaged on the FMBIO II.

Paternity testing was demonstrated by employing fluorescent imaging of PBXL-1 in a multiplexed GBA microarray format (Fig. 8B). Compared to the MTI predicate device [11], there were no false-positive or false-negative results. All of these single nucleotide polymorphisms were recognized with a signal-to-background ratio of between 30:1 and 5:1 with this system. The sequence of the polymorphic sites was determined previously by a sequencing procedure [11]. This information was used to characterize the occasional anomalous signals present on the arrays in Fig.8 as target template- and polymerase-dependent phenomena. These anomalous signals were also present in the predicate device and therefore not a function of the PBXL detection system. The results for the 10× PCR/GBA found no exclusion for the family. However, for the placental DNA (unrelated control), an exclusion was found in Locus 210, which demonstrates the utility of the test (Table II).

The relatively high molecular weight PBXL conjugates were able to detect single nucleotide polymorphisms in a human DNA system (Fig. 8). This demonstrated that the binding of the PBXL fluors was not sterically hindered when the specific binding partner was freely accessible. The net result was an efficient, rapid, nonelectrophoretic, nonradioactive, miniaturized method for the detection of PCR products.

A fundamental limit to array miniaturization is the signal generated per unit area. Direct detection of DNA arrays typically employs R-PE. Nonetheless, its low fluorescent intensity (relative to PBXL) will limit future array miniaturization. Additional experimentation is under way to characterize the sensitivity of the PBXL-based system

Table II. Data Summary of Genetic Bit Analysis Data Shown in Fig. 8 for Single-Nucleotide Polymorphisms as a Model System

Locus	Father	Mother	Daughter	Placental DNA
12	CC	CT	CC	CC
20	AG	GG	GG	GG
21	AA	AG	AA	AA
27	TT	CT	TT	CT
45	TT	TG	TG	TG
60	CC	CC	CC	CC
84	CC	CT	CC	CT
94	TT	CC	CT	CT
210	**AA**	**CC**	**AC**	**CC**
212	AG	AG	AG	GG

versus R-PE. Subsequent optimization of the PBXL technology in this format should demonstrate a substantial sensitivity improvement relative to R-PE. This anticipated gain in sensitivity should enable a greater degree of miniaturization in microarray technologies.

APPLICATIONS TO FLOW CYTOMETRY

Flow cytometry is where PBXL dyes have great potential for enabling the discovery of new clinically important targets and improving the sensitivity of existing methods. Since individual cells are interrogated by this technique, it is impossible to provide amplification via a multistep process. A powerful direct fluorescent approach is necessary for future advancements in flow cytometric detection of low-density events. Furthermore, the installed instruments would not require modifications to capitalize on the potential provided by the PBXL dyes.

Flow cytometry routinely employs R-phycoerythrin (PE), FITC, and other fluors for the fluorescent detection of high- and medium-density white blood cell surface markers [7]. However, the fluorescent intensity of these fluors limits their utility for the detection of low-density (dim) markers. The CD56 cell surface marker for natural killer (NK) cells was evaluated because it is a dim marker that, while detected by PE, is not clearly separated from background cells. A CD56:PBXL-1 conjugate demonstrated superior sensitivity and clinical utility relative to CD56:PE conjugates when processed via the ImmunoPrep reagent system (Beckman-Coulter, Miami, FL). This work was done under contract by FAST Systems (Rockville, MD).

In this study PBXL-1 was conjugated to CD56 antibody (Beckman–Coulter) through SATA/sulfo-SMCC heterobifunctional cross-linking chemistry (Pierce) at an offered molar ratio of 18:1, respectively. Serial dilutions of the CD56:PBXL-1 conjugate were tested and compared to the recommended concentration (0.3 μg/test) of a CD56:PE control conjugate (Beckman-Coulter). Lymphocytes from one individual were previously stained with CD3:FITC (Becton–Dickinson) to aid in defining the NK cells. Cells were stained via the ImmunoPrep reagent system and analyzed on an EPICS Elite flow cytometer (Beckman-Coulter) with excitation from a 488-nm argon laser. A concentration of 1.05 μg/test of the CD56:PBXL-1 conjugate demonstrated a comparable percentage of positive cells relative to the recommended concentration of the CD56:PE conjugate. The binding kinetics of the CD56:PBXL-1 (1.05 μg/test) and CD56:PE (recommended concentration of 0.3 μg/test) conjugates were compared. The studies were performed at 4°C using lymphocytes previously stained with CD3:FITC. All other conditions were the same as in the titration study. Lymphocytes from 10 randomly selected patients were used to compare the CD56:PBXL-1 conjugate (1.05 μg/test) to the CD56:PE and NKH1:PE (Beckman-Coulter) conjugates at recommended dilutions. The study employed the ImmunoPrep reagent system and was run at 42°C for 30 min on cells previously stained with CD3:FITC.

The kinetics study was performed to estimate the impact of the large molecular weight of the PBXL dyes on the binding kinetics. The PBXL dye had no practical effect on kinetics since both conjugates reached a mean channel fluorescence plateau by 5 min (Fig. 9). It should be noted that the 488-nm argon laser excites PE and PBXL-1 at approximately 75 and 36% of maximum, respectively. Furthermore, the FL-2 emission filter (575-nm bandpass) used for PE (573 nm emission) is nearly optimal, while the FL-4 filter (675-nm bandpass) used for PBXL-1 (666 nm emission) is less than optimal. Nonetheless, the CD56:PBXL-1 conjugate had approximately five times the signal of the PE conjugate.

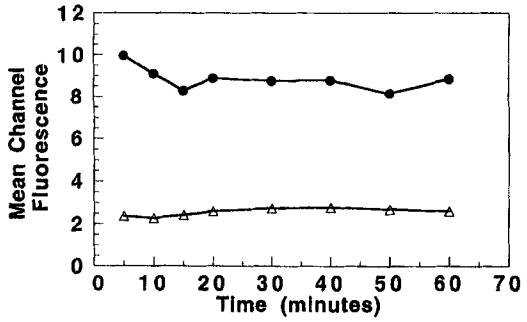

Fig. 9. Reaction kinetics of CD56:PE (△——△) and CD56:PBXL-1 (●——●) conjugates.

Table III. Ten-Patient Study Comparing Percentage Positive Cells and Mean Channel Flourescence for CD56:PBXL-1, CD56:PE, and NKH-1:PE Conjugates

	% positive			Mean flourescence		
PID	CD56:PE	NKH-1:PE	CD56:PBXL-1	CD56:PE	NKH-1:PE	CD56:PBXL-1
703414	9.7	9.8	10.7	2.23	1.58	7.57
703415	18.3	18.9	18.8	1.55	1.21	4.30
703555	3.6	12.6	12.5	1.50	1.80	6.21
703556	3.4	5.4	7.4	2.23	2.30	4.20
703557	6.5	4.2	6.3	1.16	1.53	6.65
703558	4.4	10.4	11.6	1.68	1.38	4.76
703559	7.1	9.9	11.5	0.94	1.42	4.46
703560	19.6	21.8	18.3	3.43	4.76	3.95
703561	16.3	19.9	18.7	2.09	3.45	4.47
703562	7.7	13.4	14.2	2.31	2.61	4.62

A 10-patient study was performed to estimate the impact of the high molecular weight PBXL dyes on the nonspecific staining of cells. These data demonstrate comparable nonspecific staining performance (percentage positive cells) for the CD56:PBXL-1, CD56:PE, and NKH1:PE conjugates (Table III).

A histogram comparing the distribution of stained cells achieved with CD56:PE compared to CD56:PBXL-1 in patient 703414 shows much cleaner resolution from background cells for the PBXL-1 detection method (Fig. 10). Many CD56 conjugates have difficulty in resolving positively from negatively stained cells. PBXL-1 offers a distinct advantage over R-PE in the detection of the low-density marker CD56.

The fluorescence intensity of conventional fluors has limited their utility for the detection of low-density (dim) cell surface markers. PBXL-1 demonstrated superior sensitivity over PE for detecting the dim marker CD56. Clinical utility was improved due to enhanced resolution of positive from negative cells. It should be possible to employ the PBXL dyes for sensitive detection of other dim markers and ultradim markers. Furthermore, the opportunity now exists for the discovery of high-value cell surface markers previously undetectable using conventional fluors.

SUMMARY

The applications for PBXL dyes summarized in this paper demonstrate four key performance characteristics:

First, PBXL technology can be formatted in assays that have a low nonspecific signal. The femtomolar sensitivity demonstrated in the TSH assay was achievable in part because of the low nonspecific binding of the PBXL conjugate. The low background in blotting applications is a major advantage.

Second, the size of the PBXL dyes does not impact reaction kinetics in solution. Anti-CD56 conjugates to PBXL and PE both demonstrated the maximum signal after a 5-min incubation. Rapid reaction times are also possible in solid phase applications such as blotting.

Third, the size of the PBXL fluors was not a steric impediment where the antigen or specific binding partner was freely accessible on a solid phase. The streptavi-

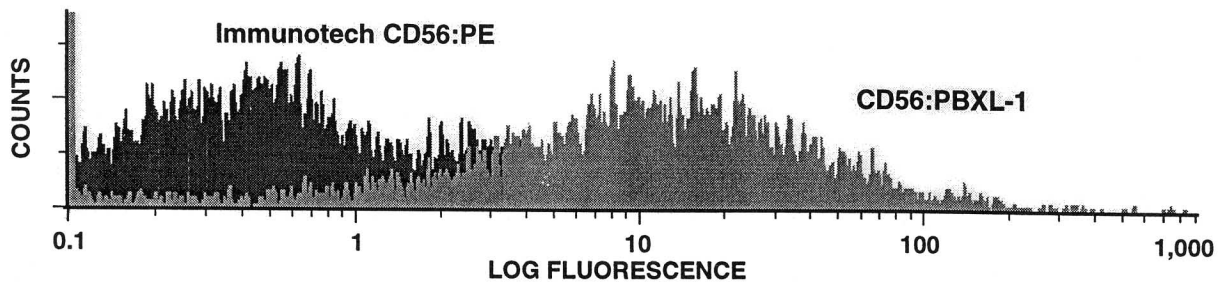

Fig. 10. The distribution of lymphocytes stained with CD56:PE and CD56:PBXL-1 conjugates for patient 703414.

din:PBXL conjugate was able to detect single-nucleotide polymorphisms when formatted in a DNA array.

Fourth, PBXL dyes demonstrated a high sensitivity without the need for additional signal amplification or signal triggering steps.

These attributes make the PBXL technology poised to address two of the major challenges to the medical diagnostic industry, enhanced clinical sensitivity and assay miniaturization. A detection technology that dramatically improves the sensitivity of existing tests will enable earlier disease detection. This is critical for the diagnosis and therapeutic monitoring of diseases such as AIDS and cancer. Moreover, a highly sensitive detection system will enable discovery of clinically important, previously undetectable disease markers. PBXL technology demonstrated superior sensitivity over conventional direct detection technologies in clinically relevant applications. Furthermore, these data were generated on instrumentation not ideally suited to the attributes of the PBXL dyes. We project significant sensitivity improvements, as instrumentation is developed to take advantage of the attributes of the PBXL technology.

The increasing need for faster assay throughput and lower test costs is the major motivation behind assay miniaturization of immunodiagnostic and nucleic acid-based diagnostic testing. High-density arrays simultaneously run large test numbers in a small area, thereby consuming less reagent per test and improving assay throughput. One of the basic limitations to assay miniaturization is the signal per unit area. The PBXL dyes are the brightest direct fluors available and should enable greater sensitivity for higher-density miniaturized arrays.

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